8-2009

Characterization of the Toc complex by blue native PAGE:oligomeric and dynamic changes of the Toc complex

William I. Crenshaw
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To the Graduate Council:

I am submitting herewith a thesis written by William I. Crenshaw entitled "Characterization of the Toc complex by blue native PAGE:oligomeric and dynamic changes of the Toc complex." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Barry D. Bruce, Major Professor

We have read this thesis and recommend its acceptance:

Gladys Alexandre, Beth C. Mullin

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis Presented for
Master of Science
Degree
The University of Tennessee, Knoxville

William I Crenshaw
August 2009

ACKNOWLEDGEMENTS

First, I would like to thank my family for their constant love, encouragement, and support. In this aspect of my life, I have been absolutely blessed. I would like to thank my friends and roommates for their company, support, and for being there whenever needed. I would like to thank my lab-mates for their friendship, help, and for their sharing of knowledge. In particular, I would like to thank Michael Vaughn for volunteering his general knowledge of everything scientific and/or mechanical. Evan Reddick was very helpful with advice for using nucleotides and has always been willing to critically read any manuscript presented. Richard Simmerman produced the POTRA1 construct utilized in this work. Ian Campbell allowed me to use his general import figure. I would also like to thank Ashita Dave and Prakitchai (Non) Chotewutmontri for their help. Dr. John Biggerstaff was a huge help with the TIRF microscopy and data analysis. I would like to thank my PI Barry Bruce for his encouragement and support, particularly when I was the closest to giving up. I am very grateful for my wonderful committee members Gladys Alexandre and Beth Mullin and would like to thank them for their help and for continuing to be there throughout the many holdups and postponements. Finally, I would like to thank my wonderful sister-in-law for giving me the small push that I needed to apply to the Master Brewers Program.

ABSTRACT

The majority of chloroplast proteins are nuclear encoded and transcribed on cytosolic ribosomes, and therefore must be post-translationally imported into the chloroplast. Preproteins are directed to the chloroplast via a cleavable N-terminal extension known as a transit peptide. This transport is mediated by the Toc and Tic complexes (Translocon at the Outer/Inner Chloroplast envelope membrane), functioning in tandem to transport preproteins into chloroplasts relying on the hydrolysis of ATP and GTP. The Toc complex is composed of the β-barrel channel protein Toc75 and the homologous GTPase receptors Toc34 and Toc159. GTP hydrolysis is necessary for the formation of the early import intermediate, in which the transit peptide is inserted into the Toc channel, but the presence of internal ATP is the only energetic requirement for the later stages of translocation to occur, mediated by the stromal motor complex with an Hsp100 isoform hydrolyzing stromal ATP. The purpose of the current study is to characterize the change in stability and/or oligomeric status of the Toc complex with the incubation of nucleotides, analogs, proteins/peptides, etc. by blue native electrophoresis followed by 2d SDS-PAGE. The Toc complex ranges from ~800 kDa to greater than 1320 kDa for the proposed Toc/Tic supercomplex when no proteolytic degradation has occurred. Proteolytic degradation of Toc159 is correlated with the appearance of complexes with a mass ranging from 800 kDa to 440 kDa and below. Proteolytic degradation of Toc159 is more apparent in chloroplasts purified from older Pisum sativum plants. The results of the incubation of chloroplasts with GDP, GTP, and non-hydrolyzable analogs before analysis by 2d electrophoresis followed by western blot hybridization suggest that the loading of the GTPase receptors with nucleotide triphosphate results in the increased association of Toc components in complexes in the size range of 880-630 kDa.

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CHAPTER I

1.0 INTRODUCTION AND GENERAL INFORMATION

1.1 Origin of Plastids

The primary feature that distinguishes plants from animals is their reliance on solar energy to generate molecules with energy rich bonds in the process of photosynthesis, on which almost all life on Earth depends, either directly or indirectly. Chloroplasts constitute the site of oxygenic photosynthesis in plants. The chloroplast is the most studied member of the plastids, which have a prokaryotic origin. Between 1.2 and 1.5 billion years ago (Ga), a single celled proto-eukaryote containing mitochondria (from a previous endosymbiotic event > 1.5 Ga) engulfed and formed a symbiotic relationship with a photosynthetic cyanobacterium, giving rise to the plastids (Dyall, Brown et al. 2004). These two endosymbiotic events left the eukaryotic cell with compartmentalized bioenergetic and biosynthetic capacities (Dyall, Brown et al. 2004).

Genome reduction played a key role in the transition from autonomous endosymbiont to organelle. Over the course of evolutionary time, the chloroplast genome was reduced orders of magnitude, from the size of a eubacterial genome to roughly the size of plasmids (Timmis, Ayliffe et al. 2004). During this transition, the plastids have largely retained their prokaryotic biochemistry (Timmis, Ayliffe et al. 2004). Many genes of cyanobacterial origin have been lost, and the majority of the genes retained have been transferred to the host nucleus (Martin, Rujan et al. 2002). These genes must have existed in duplicate until a targeting mechanism evolved to relocate the gene products to the plastid (Dyall, Brown et al. 2004). Greater than 90% of mitochondrial and chloroplastic proteins are nuclear encoded and synthesized on cytosolic ribosomes as preproteins containing a cleavable N-terminal targeting sequence, called a presequence or transit peptide for mitochondria and chloroplasts, respectively (Bhushan, Kuhn et al. 2006). The proteins destined for the matrix or stroma must be post-translationally imported across the double membrane systems which are

present in both of these classes of organelles. In the chloroplast, this translocation is accomplished by the Translocon at the Outer Chloroplast envelope membrane (Toc complex) and the Translocon at the Inner Chloroplast envelope membrane (Tic complex) (Soll and Schleiff 2004; Kessler and Schnell 2006; Jarvis 2008). Translocation is accomplished by the analogous Tom and Tim complexes in the mitochondria (Bohnert, Pfanner et al. 2007; Neupert and Herrmann 2007), but the main components are not closely related (Jarvis 2008).

1.2 Gene Transfer and Protein Import

Chloroplasts contain a remnant of their ancestral genome, comprised of between 120 and 135 genes, ~75 coding for proteins, and the rest for other RNAs (Lopez-Juez and Pyke 2005). Most of the chloroplast genome encodes components of thylakoid photosynthetic complexes and the machinery necessary for their translation and assembly (Lopez-Juez and Pyke 2005). Chloroplasts have a high rate of transcription and translation, which allows the production of large amounts of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and supports the high turnover rate of electron transfer components, which are necessary for efficient carbon fixation (Leister 2003). On the other hand, the majority of chloroplast proteins are encoded in the nucleus, transcribed on cytosolic ribosomes, and are imported into the chloroplast post-translationally (Keegstra and Cline 1999). N-terminal extensions, named transit peptides, which are cleaved after/during late stages of import contain the information necessary for the targeting of these preproteins to the chloroplast, which may be mediated by cytosolic chaperones (Bruce 2000; Bruce 2001).

The chloroplast is a complex organelle, with three membrane systems (two envelope membranes and the internal thylakoid membranes) and 6 possible locations for targeting of preproteins (each of the membranes, the intermembrane space, the stroma, and the thylakoid lumen). Most chloroplast proteins are translocated across the envelope membranes in their precursor form, and the transit peptide is cleaved by the stromal processing peptidase,

yielding the mature form of the protein (Jarvis 2008). This translocation is mediated by multimeric protein complexes at the envelope membranes named Toc and Tic (Translocon at the Outer/Inner envelope membrane of Chloroplasts). The individual components of these complexes are named with Toc or Tic, depending on the membrane association, followed by the molecular mass in kilodaltons, and the two letter prefix representing the species of origin can be included (*P. sativum* is implied if no prefix is used) (Schnell, Blobel et al. 1997). The Toc complex is composed of the channel protein Toc75, the GTPases Toc34 and Toc159 (Toc86 is a proteolytic fragment of Toc159), as well as Toc64 and Toc12 which have been proposed to have roles in chaperone recruitment (Soll and Schleiff 2004; Kessler and Schnell 2006; Jarvis 2008). The Tic complex has not been as well characterized, but its components include Tic110, Tic40, Tic22, and Tic20 (Jarvis 2008). After translocation, the transit peptide is cleaved, and proteins are further targeted to their final location (Keegstra and Cline 1999).

1.3 Translocons

Almost half of an average cell's proteome is inserted into or transported across a biological membrane, facilitated by translocons (Schatz and Dobberstein 1996). Translocons are remarkably flexible hetero-oligomeric complexes, which not only allow hundreds or thousands of distinct protein substrates to cross the bilayer while maintaining the permeability barrier, but many are able to sense stop-transfer signals and integrate membrane proteins laterally into the bilayer (Schnell and Hebert 2003). Translocons are generally composed of a targeting system on the cis face of the membrane, a hetero-oligomeric transmembrane channel, and a peripherally associated molecular motor powered by nucleoside triphosphates (Schatz and Dobberstein 1996). Proteins can be translocated either cotranslationally or posttranslationally, and contain a signal sequence to direct the protein to its final destination (Blobel 1980). Amino-terminal targeting signals are used in both prokaryotes and eukaryotes to direct proteins from their site of translation to their final destination,

which can include the bacterial inner membrane, as well as the ER, mitochondria, or chloroplasts in eukaryotes (Jarvis 2008). The most common class is the signal-gated translocons, which are related to gated ion channels and transport nascent or newly synthesized precursors in a largely unfolded conformation with the help of molecular chaperones, and include the *E. coli* SecYEG, ER Sec61, Thylakoid cpSec, and the translocons of the double membrane systems of the mitochondria and chloroplast (Schnell and Hebert 2003). The second class is the signal-assembled translocons, able to accommodate large, fully folded proteins or oligomeric protein complexes, which include the peroxisome PEX system, as well as the *E. coli* TAT and the thylakoid TAT/ΔpH systems (Schnell and Hebert 2003).

1.4 Precursors and Transit Peptides

Greater than 90% of mitochondrial and chloroplastic proteins are nuclear encoded and synthesized on cytosolic ribosomes as preproteins containing a cleavable N-terminal signal peptide, referred to as a presequence in mitochondria and a transit peptide in chloroplasts (Bhushan, Kuhn et al. 2006). Transit peptides are removed by the stromal processing peptidase (SPP) during translocation across the inner envelope membrane. Proteins destined for the thylakoid lumen contain bipartite transit peptides, with the N-terminal portion containing the stromal targeting information, which is removed in the stroma, and the C-terminal portion containing the thylakoid targeting information (Tranel and Keegstra 1996). Transit peptides are both necessary (Pilon, Wienk et al. 1995; Rensink, Pilon et al. 1998) and sufficient (Smeekens, van Steeg et al. 1987) to direct the target protein into the chloroplast stroma with high specificity; however, transit peptides do not contain any regions of highly conserved amino acids (von Heijne, Steppuhn et al. 1989). Transit peptides are enriched in hydrophobic (Ala, Leu, Phe, Val), hydroxylated (Ser, Thr), and positively charged residues (Arg, Lys), and deficient in acidic residues, and they vary greatly in length (13-146 residues, mean 58, with most being 30-80) (Zhang and Glaser 2002).

Hydroxylated residues participate in a loose phosphorylation motif (Waegemann and Soll 1996), which allows phosphorylated preproteins to interact with the guidance complex containing 14-3-3 proteins and an Hsp70 homologue (May and Soll 2000).

Transit peptides are largely unstructured in an aqueous environment (Wienk, Czisch et al. 1999; Bruce 2000), which supports an early hypothesis that transit peptides have evolved to maximize their random coil nature, which could promote their interaction with chaperones (von Heijne and Nishikawa 1991). Transit peptides have been shown to specifically interact with lipids present in the outer envelope membrane (van't Hof, van Klompenburg et al. 1993; Pilon, Wienk et al. 1995; Pinnaduwage and Bruce 1996), and this interaction has been shown to induce the formation of N and C-terminal α-helical regions in the transit peptide (Horniak, Pilon et al. 1993; Wienk, Czisch et al. 1999; Wienk, Wechselberger et al. 2000). Membrane induced secondary structure has been postulated to play a role in transit peptide recognition by the receptors of the Toc complex (Bruce 2000). Transit peptides do not contain highly conserved homology blocks; however, stromal-targeting transit peptides do contain three distinct regions: an uncharged N-terminal domain enriched in hydroxylated residues, a central domain lacking acidic residues, and a C-terminal domain enriched in arginines (von Heijne, Steppuhn et al. 1989). The N-terminal region is important for the interaction between the transit peptide and the envelope lipid MGDG, the middle region seems to function as a flexible connector region, containing proline, turn promoting residues (G and N), as well as the semi-conserved FGLK motif, and the positively charged C-terminal region may interact with membrane lipids through electrostatic interactions (Pilon, Wienk et al. 1995; Wienk, Czisch et al. 1999).

1.4.1 Transit Peptide Processing (cleavage and degradation)

The stromal processing peptidase (SPP) has a strong affinity for the transit peptide (Richter and Lamppa 1999), as processing can occur while the C-

terminus of the preprotein is exposed to the chloroplast exterior (Schnell and Blobel 1993). SPP contains a conserved zinc-binding motif, characteristic of the pitrilysin family of metallopeptidases, which also includes the mitochondrial processing peptidase (Richter and Lamppa 1998). Recognition by SPP depends on the C-terminal 10-15 residues of the transit peptide, which are usually characterized by a positive net charge (Richter and Lamppa 2002), and recognition may rely more on overall physiochemical properties in the cleavage region rather than on the presence of specific residues (Rudhe, Clifton et al. 2004). SPP processing releases the mature protein, but the transit peptide undergoes a second cleavage reaction, disrupting the binding site before it is released to be degraded by a distinct ATP-dependent metallopeptidase (Richter and Lamppa 1999; Richter and Lamppa 2002). The presequence protease (PreP) is also a member of the pitrilysin family of metallopeptidases, and is responsible for the degradation of transit peptide fragments in the chloroplast stroma (Moberg, Stahl et al. 2003).

1.5 General Import Pathway into Chloroplasts

Most chloroplast proteins are nuclear encoded, translated on cytosolic ribosomes as higher molecular weight precursors, and directed to the chloroplast by their N-terminal transit peptides, shown in Fig. 1-1 (Keegstra and Cline 1999). These proteins are imported into the chloroplast stroma by the coupled Toc/Tic complexes (Soll and Schleiff 2004). The plant transit peptide averages 58 residues in length (Zhang and Glaser 2002) and is both necessary (Pilon, Wienk et al. 1995; Rensink, Pilon et al. 1998) and sufficient (Smeekens, van Steeg et al. 1987) to direct proteins to the chloroplast. Proteins that are transcribed on cytosolic ribosomes may be targeted to the chloroplast by several different routes before engaging the general import pathway (Jarvis 2008). The first possibility is the association of the phosphorylated preprotein with a guidance complex containing 14-3-3 proteins and an Hsp70 protein (May and Soll 2000). This guidance complex most likely hands off the preprotein to Toc34, since Toc159

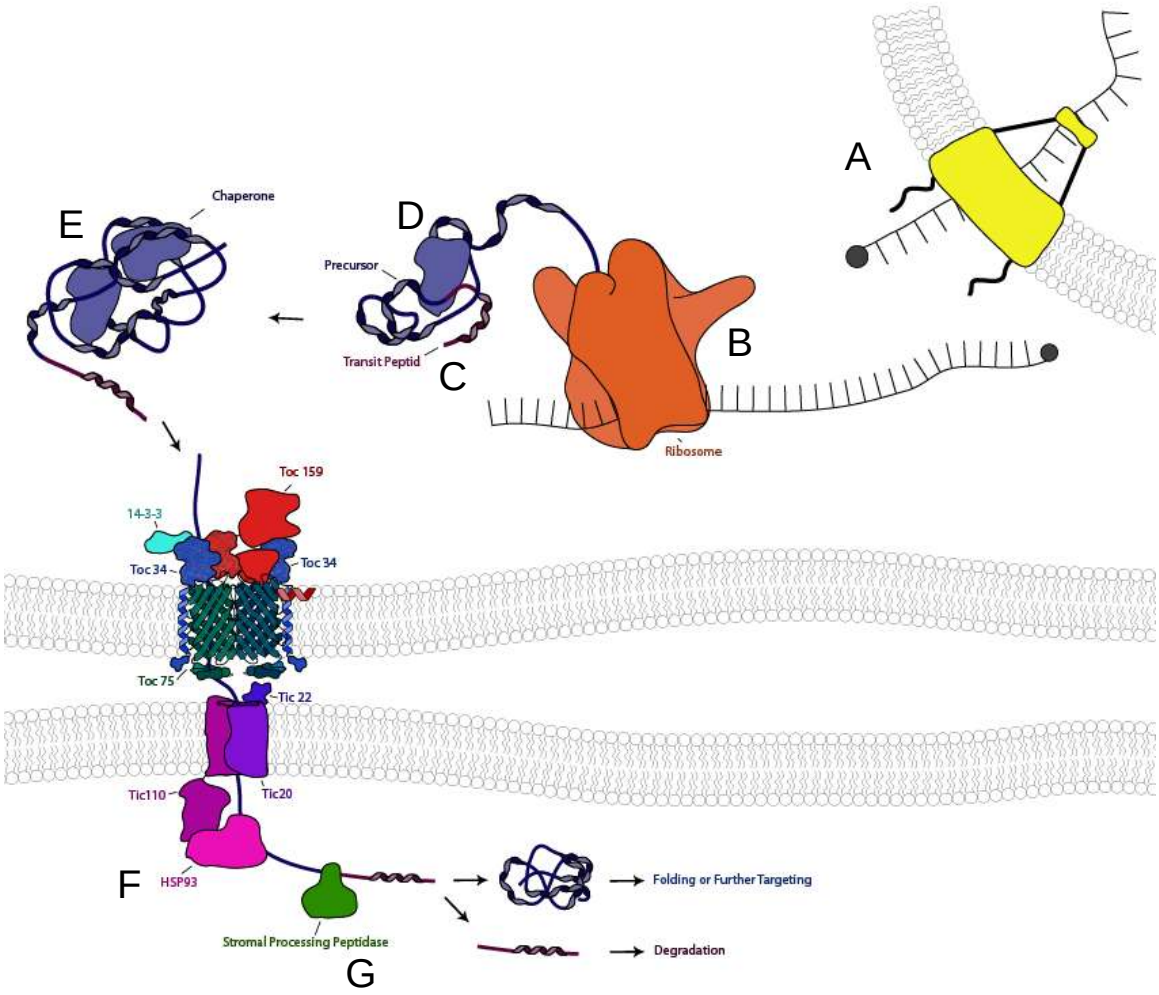


Figure 1-1 Model of general import pathway of preproteins into chloroplasts.

Most chloroplast proteins are encoded in the nucleus (A), transcribed on cytosolic ribosomes (B) and post-translationally imported into the chloroplast. The proteins are transcribed as preproteins with a cleavable, N-terminal transit peptide (C). Nascent chains interact with cytosolic chaperones to prevent aggregation and misfolding (D). Preproteins are guided to the Toc complex by chaperones such as hsp70, hsp90, or 14-3-3 proteins (E). The transit peptide is recognized by the GTPase receptors Toc159 and Toc34 at the chloroplast surface, and the early import intermediate forms in which the transit peptide is inserted into the β-barrel channel protein Toc75. Translocation occurs through the Toc and Tic complexes simultaneously at contact sites between the outer and inner envelope membranes, and Hsp93 functions as the ATP fueled stromal motor providing the driving force for translocation (F). During translocation the transit peptide is cleaved by the Stromal Processing Peptidase (G) and the protein is folded with the help of stromal chaperones, or is further targeted to its final destination. Adapted from (Reddick, Chotewutmontri et al. 2008).

does not recognize phosphorylated preproteins (Becker, Jelic et al. 2004). The second possibility is preprotein association with a cytosolic Hsp90 which guides the preprotein to Toc64, which has been postulated to hand off the preprotein to Toc34 after ATP dependent dissociation of the chaperone (Qbadou, Becker et al. 2006). A third possibility is the direct association of transit peptides with outer envelope lipids, inducing α-helical secondary structure which may aid their recognition by one of the GTPase receptors of the Toc complex (Bruce 2000). It has been postulated that a soluble form of Toc159 functions in shuttling preproteins to the Toc complex (Hiltbrunner, Bauer et al. 2001; Bauer, Hiltbrunner et al. 2002; Smith, Rounds et al. 2004); however, this seems to be an experimental artifact (Becker, Jelic et al. 2004), which will not be discussed in detail in the present work. Several outer envelope proteins are translated in the cytosol without a transit peptide and are inserted in the membrane without the requirement of other proteins or nucleotides (Schleiff and Klosgen 2001). One other possibility is the direct binding of preproteins to the Toc complex.

Once the preprotein has reached the surface of the chloroplast, translocation usually occurs by the combined actions of the Toc and Tic complexes at contact sites between the two membranes (Schnell and Blobel 1993). Based on energetic requirements determined by *in vitro* cross-linking and immunoprecipitation experiments, chloroplast protein import can be divided into three stages, although *in vivo* preproteins most likely proceed through these steps seamlessly due to the lack of energetic constraints (Jarvis 2008). The initial reversible binding or recognition of the transit peptide by the Toc complex is an energy independent step (Perry and Keegstra 1994; Ma, Kouranov et al. 1996; Kouranov and Schnell 1997). In the absence of nucleotide, preprotein interacts with at least Toc159, as shown by label transfer cross-linking (Perry and Keegstra 1994). This interaction was shown to be productive by chasing the preprotein into an active translocation complex by the addition of ATP (Perry and Keegstra 1994). After energy independent binding, the early import intermediate is formed, which is characterized by insertion of the transit peptide into the Toc75

channel, where it interacts with components of the Tic complex (Ma, Kouranov et al. 1996; Nielsen, Akita et al. 1997). Formation of the early import intermediate is irreversible, and relies on low levels of ATP (~100 μM) in the intermembrane space as well as GTP (Olsen and Keegstra 1992; Kessler, Blobel et al. 1994; Young, Keegstra et al. 1999). The final stage of import is translocation, and is characterized by a high requirement for ATP (> 1 mM) in the stroma (Theg, Bauerle et al. 1989). The transit peptide is cleaved by the stromal processing peptidase as it emerges into the stroma, before translocation is complete (Richter and Lamppa 1999).

1.6 Toc Apparatus

Research in chloroplast protein import has been ongoing for more than 30 years (Dobberstein, Blobel et al. 1977); however, the identification of components of the import apparatus and the assignment of function to these components has only been seen in the last 2 decades (Waegemann and Soll 1991; Perry and Keegstra 1994; Schnell, Kessler et al. 1994). Waegemann and Soll (1991) identified the receptor Toc159 (Toc86) and an Hsp70 chaperone of the Toc complex by the specific binding of precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase (preSSU) by isolated outer envelopes of pea chloroplasts. Perry and Keegstra (1994) identified Toc159 (Toc86) and Toc75 by label transfer cross-linking from prSSU to an arrested early import complex. Kessler *et al*. (1994) identified the two receptor proteins Toc34 and Toc159 (Toc86) as GTP binding proteins by analyzing cDNA clones and demonstrating the inhibition of preprotein translocation by non-hydrolyzable GTP analogs. In recent years, several other Toc components have been identified.

1.6.1 The Toc Subunits

1.6.1.1 Toc75

Toc75 was initially identified by label transfer cross-linking from radiolabeled prSSU bound to the Toc complex in intact *P. sativum* chloroplasts in

the presence of low levels (75 μM) of ATP (Perry and Keegstra 1994). In the absence of exogenous ATP, only Toc86 was labeled, suggesting the nucleotide dependence of the transfer of preprotein from initial receptor to contact sites involving other Toc and Tic components (Perry and Keegstra 1994). Toc75 was identified as an integral membrane protein by resistance to thermolysin digestion, salt and high pH extraction, and was suggested to form a membrane spanning β-barrel channel (Schnell, Kessler et al. 1994). Electrophysiological measurements were conducted on heterologously expressed Toc75 reconstituted into a planar lipid bilayer, showing a narrow restriction zone functioning as a selectivity filter and a wider vestibule area with diameters of ~14Å and 26Å, respectively (Hinnah, Wagner et al. 2002). A previous study by the same group showed that Toc75 forms a voltage-gated transmembrane channel with a hydrophilic pore and a precursor binding site (Hinnah, Hill et al. 1997). This precursor binding site showed a preference for genuine transit peptide over a mitochondrial presequence or a related synthetic peptide with a similar charge but different structure (Hinnah, Wagner et al. 2002). Analysis of the *Arabidopsis* genome shows the presence of 3 genes with significant homology to psToc75; however, AtToc75-III is the only gene that encodes a protein large enough to be considered a functional homologue of psToc75 (Jackson-Constan and Keegstra 2001). Toc75 shows a high level of similarity to SynToc75 from *Synechocystis* PCC6803 (22% identity, 55% similarity), and the reconstituted protein forms a channel with properties similar to Toc75 (Bolter, Soll et al. 1998). SynToc75 is localized in the outer membrane and is related to a group of prokaryotic secretion channels which export virulence factors across the outer membrane (Reumann, Davila-Aponte et al. 1999). The sequence and functional similarity and outer membrane localization suggest that SynToc75 may have played a significant role in the establishment of the endosymbionic protein import system (Bolter, Soll et al. 1998). Toc75 is a member of the Omp85 family of proteins, which are characterized by a C-terminal integral membrane β-barrel domain and an N-terminal domain rich in polypeptide-transport-associated (POTRA) repeats

(Gentle, Burri et al. 2005). Toc75 contains three POTRA domains, which are predicted to contain three β-strands with the latter two separated by two α-helices (Sanchez-Pulido, Devos et al. 2003). These domains may have a chaperone-like function, binding transit peptides before their translocation through the pore (Ertel, Mirus et al. 2005). An N-terminal region of Toc75 may also be involved in complex formation with Toc34 (Ertel, Mirus et al. 2005).

1.6.1.2 The Small Toc GTPases

Toc34 was identified by the solubilization of the import machinery interacting with preprotein in the early import intermediate stage (Schnell, Kessler et al. 1994). A cDNA clone was generated which exhibited GTP binding capability, integration in the outer chloroplast membrane via a C-terminal helical anchor, and the projection of a large N-terminal domain on the chloroplast surface (Kessler, Blobel et al. 1994). Toc34 has been shown to be involved in early import intermediates with preproteins along with Toc159 and Toc75 by co-immunoprecipitation (Nielsen, Akita et al. 1997) and by several different cross-linking strategies (Ma, Kouranov et al. 1996; Akita, Nielsen et al. 1997; Kouranov and Schnell 1997). GTPγS and GMP-PNP, slowly hydrolyzable and nonhydrolyzable GTP analogs, respectively, have been shown to inhibit the formation of early import intermediates in *in vitro* import assays (Olsen and Keegstra 1992; Kessler, Blobel et al. 1994; Young, Keegstra et al. 1999); however, Becker *et al.* (2004) showed that GMP-PNP stimulated the binding of preprotein to isolated Toc34. The addition of GTP increases the affinity of Toc34 for preproteins, and GDP loaded Toc34 has a decreased ability to bind preproteins (Sveshnikova, Soll et al. 2000; Schleiff, Soll et al. 2002). These results may imply the necessity for binding and hydrolysis of GTP for the progression from energy independent binding to the early import intermediate involving insertion of the preprotein into the translocation channel to occur (Kouranov and Schnell 1997). The presence of GMP-PNP eliminated the label transfer cross-linking of Toc34 during energy independent binding of preproteins,

which could be explained by the nucleotide-dependent regulation of the association of Toc34 with other Toc components or a GTP induced conformational change in the Toc complex which is necessary for the insertion of the preprotein in the protein conducting channel of the outer membrane (Kouranov and Schnell 1997).

The *Arabidopsis thaliana ppi1* mutant (plastid protein import) was identified by a screen of T-DNA insertion mutants, and is characterized by a pale phenotype for the first two weeks of life, and a wild-type appearance of mature leaves during later stages of development (Jarvis, Chen et al. 1998). The insertion disrupts a gene encoding a 33 kilodalton homologue of atToc34, which revealed the presence of two isoforms of Toc34 (61% amino acid identity) in *Arabidopsis thaliana* (Jarvis, Chen et al. 1998). Two lines of antisense plants were generated with reduced expression of the two Toc34 homologues, and the α-atToc34 plants showed a much milder phenotype (Gutensohn, Schulz et al. 2000). Jarvis *et al.* (1998) overexpressed cDNA clones of atToc33 or atToc34 in a *ppi1* background, and either protein was able to complement the deficiency, showing a high level of functional redundancy between the two isoforms. Kubis *et al.* (2003) have shown the deficiency of *ppi1* chloroplasts of photosynthetic proteins, and have suggested the preferential import of photosynthetic proteins by atToc33 containing Toc complexes, and the preference for non-photosynthetic, housekeeping proteins by atToc34. Two Toc34 homologues have been found in many other species as well, including spinach, rape seed, potato, tomato, poplar, maize, and three isoforms in the moss *Physcomitrella*; however, the isoforms are more identical in most of these species than atToc33 and atToc34 (Voigt, Jakob et al. 2005).

Toc34 can be phosphorylated *in vitro* and *in vivo* by a kinase associated with the outer chloroplast envelope membrane (Sveshnikova, Soll et al. 2000; Jelic, Sveshnikova et al. 2002). This phosphorylation results in a decreased affinity of Toc34 for GTP and preprotein (Sveshnikova, Soll et al. 2000). Phosphorylation also inhibits the association of Toc34 with other members of the

Toc complex (Oreb, Hofle et al. 2008). On the other hand, GTP loaded non-phosphorylated Toc34 has an increased affinity for phosphorylated preprotein (Sveshnikova, Soll et al. 2000; Schleiff, Soll et al. 2002). Therefore, the phosphorylation state regulates the GTP binding properties of Toc34, which modulates the affinity of Toc34 for preprotein. atToc33 can be phosphorylated *in vitro*, as can its pea homologue, but atToc34 cannot be phosphorylated (Jelic, Soll et al. 2003).

The crystal structure of Toc34ΔTM was solved, showing a dimer complexed with GDP and Mg^{2+} (Sun, Forouhar et al. 2002). The overall fold of Toc34 is closely related to the Ras family of GTP binding proteins; however, the Toc34 G-domain is significantly larger than most members of this family (Kessler and Schnell 2002). The Ras family consists of a large group of ~21 kDa GTP binding proteins, appearing to function in eukaryotic transmembrane signaling (Kjeldgaard, Nyborg et al. 1996). These proteins are active in their GTP bound state, interact with GTP activating proteins (GAPs), and this interaction stimulates GTP hydrolysis and the transition to the inactive GDP bound state, functioning as a "molecular switch" (Kjeldgaard, Nyborg et al. 1996). Nucleotide exchange from GDP to GTP is an intrinsically slow process which is accelerated by guanine nucleotide exchange factors (GEFs) (Vetter and Wittinghofer 2001). One of the most interesting features of the Toc34 crystal structure is the dimer interface, which shows structural similarity to the interface between a Ras-like GTP binding protein and its GAP (Kessler and Schnell 2002). Arg133 of one monomer is deeply inserted into the nucleotide binding pocket of the other monomer, which mimics the Arginine finger of Ras-like GTPases, usually supplied by either the protein itself or its GAP (Sun, Forouhar et al. 2002). The GTP regulated dimerization of Toc34 may play a role in the commitment of preproteins to translocation after binding, and nucleotide exchange may require dimer dissociation or a conformational change since a nucleotide exit site is not visible in the crystal structure (Kessler and Schnell 2002).

In addition to the presence of Toc34ΔTM as a dimer in the crystal, the presence of dimeric Toc34ΔTM has also been observed in solution by gel filtration (Sun, Forouhar et al. 2002). This group has also generated the Toc34-R128A (substitution for Arg involved in H-bonding at dimeric interface) mutant, and this mutant was characterized to exist solely in the monomeric form in solution, and to have a reduced activity even though it has a similar substrate binding affinity to wild-type Toc34 (Sun, Forouhar et al. 2002). atToc34 and atToc33 are able to dimerize, with the tightest binding occurring in the heterodimer in the presence of GDP, in the presence of GTP the homo and heterodimers showed a similar binding efficiency (Jelic, Soll et al. 2003). Reddick *et al.* (2007) have shown the concentration dependence of Toc34 dimerization; however, *in vitro* GTP hydrolysis assays did not show an increase in hydrolytic rate with the increased dimerization. Weibel *et al.* (2003) have shown that Toc33-R130A (substitution for proposed arginine finger) is limited in its ability to dimerize, is able to bind GTP and GDP with similar affinity to wild-type Toc33, and is also able to hydrolyze GTP with similar catalytic constants to wild-type. However, Yeh *et al.* (2007) have shown that atToc33 has a significantly higher GTPase activity in the dimeric form, and that storage of the protein for 1 week (4 ºC or -20 ºC) results in the sole presence of the monomeric form. Therefore, the nucleotide dependent dimerization of Toc34 may play a significant role in the regulation of preprotein recognition, GTP hydrolysis, and initiation of translocation.

1.6.1.3 The Acidic Toc GTPases

1.6.1.3.1 Toc159

The GTPase receptor Toc159 has a tripartite structure containing a protease ultra-sensitive N-terminal Acidic domain (A domain), a central GTPase domain (G domain), and a C-terminal 52 kDa membrane associated domain (M domain) (Chen, Chen et al. 2000). The G domain of Toc159 shows a high level of conservation with the G domain of Toc34 (Hiltbrunner, Bauer et al. 2001). The

intrinsic sensitivity of the A domain of Toc159 to cleavage by endogenous proteases led to its initial identification as the 86 kDa C-terminal fragment Toc86 (Waegemann and Soll 1991; Kessler, Blobel et al. 1994; Perry and Keegstra 1994; Schnell, Kessler et al. 1994; Bolter, May et al. 1998). Toc86 was identified through the binding of preprotein by isolated chloroplast outer envelope membranes (Waegemann and Soll 1991). Perry and Keegstra (1994) showed that Toc86 interacts with preproteins either in the presence or absence of ATP by label transfer cross-linking with ^{125}I-APT-prSSU (APDP is heterobifunctional, one end is activated by UV illumination and the other is sulfhydryl reactive and can be cleaved by the presence of reductant), and in the presence of ATP, Toc75 was also shown to interact with the preprotein. Kessler *et al.* (1994) characterized Toc86 as an integral membrane protein with the ability to bind GTP. Bölter *et al.* (1998) showed that chloroplasts containing intact Toc159 (which appears ~200 kDa on SDS-PAGE) can be purified from pea leaves by the inclusion of protease inhibitors, minimizing the time of the protocol, and ensuring that the temperature of the isolation media is maintained at 2 ºC. Chloroplasts isolated under these conditions still contain a small fraction of the 86 kDa fragment, and incubation of chloroplasts for one hour on ice or at ambient temperature yields >50% or complete degradation to the 86 kDa fragment, respectively (Bolter, May et al. 1998).

Toc159 was initially identified as the 86 kDa fragment lacking the A domain, and chloroplast import was biochemically characterized in isolated pea chloroplasts containing almost exclusively this fragment (Hiltbrunner, Bauer et al. 2001). The A domain contains ~30% Asp or Glu and has an overall negative charge (Bolter, May et al. 1998; Jackson-Constan and Keegstra 2001), which led to the suggestion that this domain may electrostatically interact with transit peptides, which are characterized by an overall positive charge and a low level of acidic residues (von Heijne and Nishikawa 1991). Chen *et al.* (2000) have suggested that the A domain may participate in protein-protein interactions due to its lack of sequence conservation and repetitive nature. Bölter *et al.* (1998)

have shown that import of preprotein into chloroplasts with intact Toc159 yields two fold more mature protein than chloroplasts with the 86 kDa fragment. However, Smith et al. (2004) have shown that preproteins interact with the G domain and not with the A domain by cross-linking the preprotein to Toc159, followed by selective proteolysis. Therefore, the function of the A domain remains unknown, but it has been postulated to play a role in determining preprotein binding specificity (Bauer, Chen et al. 2000).

Toc159 has been shown to exist in a stable complex with Toc75 and Toc34, even in the absence of preproteins (Waegemann and Soll 1991; Ma, Kouranov et al. 1996; Nielsen, Akita et al. 1997; Schleiff, Soll et al. 2003). However, several studies have suggested an alternative, soluble function of Toc159, interacting with preproteins in the cytosol and shuttling them to the Toc complex, possibly interacting with Toc34, and handing off preproteins to Toc75 (Hiltbrunner, Bauer et al. 2001; Bauer, Hiltbrunner et al. 2002; Smith, Rounds et al. 2004). A study by Becker *et al.* (2004) has shown that these conclusions may not have much physiological relevance because this soluble population of Toc159 is most likely an experimental artifact of Toc159 associated with low density membrane shreds from partially disrupted chloroplasts. Like most chloroplast outer envelope proteins (Schleiff and Klosgen 2001)(with the notable exception of Toc75) (Tranel, Froehlich et al. 1995; Tranel and Keegstra 1996), Toc159 is not transcribed as a higher molecular weight preprotein with a cleavable N-terminal transit peptide (Schleiff and Klosgen 2001), but is targeted to the chloroplast membrane and directly inserted via the M domain (Muckel and Soll 1996). This insertion depends on the G domain mediated interaction of Toc159 with Toc34 (Hiltbrunner, Bauer et al. 2001; Bauer, Hiltbrunner et al. 2002). The interaction between the G domains is stabilized by the presence of GDP (Smith, Hiltbrunner et al. 2002), and the presence of Toc75 along with Toc34 stimulates the insertion of Toc159 into the membrane (Wallas, Smith et al. 2003). Sun *et al.* (2002) published the crystal structure of Toc34ΔTM, and noted the conservation of residues important for GTP binding and dimerization between

Toc34 and Toc159. Yeh *et al.* (2007) have shown that dimeric psToc159G has a significantly higher GTPase activity than the monomeric form of the protein. Therefore, the homo and heterodimerization of Toc159 and Toc34 may play a role in the targeting of newly transcribed receptors to the chloroplast surface, as well as, a role in the regulation of the translocation of preproteins through the Toc complex.

Toc159 has been shown to interact with preproteins during the early stages of import (Perry and Keegstra 1994; Ma, Kouranov et al. 1996; Kouranov and Schnell 1997) and anti-Toc159 antibodies were shown to inhibit the import of preproteins (Hirsch, Muckel et al. 1994); therefore, several authors have proposed Toc159 to be the primary receptor of the Toc complex, with Toc34 playing an accessory, regulatory role (Chen, Chen et al. 2000; Smith, Rounds et al. 2004). Toc159 requires the presence of nucleotide for preprotein binding; however, binding is similar with GTP, GDP, or GMP-PNP (Smith, Rounds et al. 2004). The studies proposing a soluble function of Toc159 shuttling preproteins from the cytosol to the chloroplast surface, with the hetero G domain mediated interaction between Toc159 and Toc34 leading to the integration of Toc159 into the chloroplast outer envelope membrane, also support the role of Toc159 as the primary receptor of the Toc complex (Hiltbrunner, Bauer et al. 2001; Bauer, Hiltbrunner et al. 2002; Smith, Hiltbrunner et al. 2002; Wallas, Smith et al. 2003). However, an alternative function of Toc159 has been proposed, with Toc34 functioning as the primary preprotein receptor and Toc159 functioning as a GTP driven motor in a novel mechanism (Schleiff, Jelic et al. 2003; Becker, Jelic et al. 2004). Schleiff *et al.* (2003) have shown that preSSU is imported into proteoliposomes containing Toc75 and Toc159 in the presence of GTP, but not into proteoliposomes containing Toc75 and Toc34 (with GTP), suggesting that the minimal translocon unit is Toc75 forming the channel and Toc159 as the GTP driven motor. Becker *et al.* (2004) have shown that Toc34 binds strongly to the phosphorylated C-terminal segment of preSSU and Toc159 binds strongly to the N-terminal segment of preSSU. Under binding conditions in the presence of

GMP-PNP, only the phosphorylated C-terminal segment of preSSU reduces the binding of the competing full length preSSU, which supports the conclusion that Toc34 is the initial receptor of the Toc complex (Becker, Jelic et al. 2004). On the other hand, several studies have been published that suggest Toc159 does not have a motor function (Chen, Chen et al. 2000; Lee, Kim et al. 2003; Wang, Agne et al. 2008). Chen *et al.* (2000) have performed *in vitro* import experiments with preSSU on isolated pea chloroplasts incubated with low levels of the outer-membrane-impermeable protease thermolysin, which eliminated the A and G domains of Toc159 due to inherent proteolytic sensitivity, while leaving Toc34 and Toc75 intact. These selectively proteolyzed chloroplasts do not exhibit any energy independent binding; however, some translocation can still occur and is sensitive to the presence of non-hydrolyzable GTP analogues, demonstrating that hydrolysis at Toc34 is necessary for the initiation of translocation (Chen, Chen et al. 2000). Lee *et al.* (2003) have taken a transgenic approach, expressing the M domain of Toc159 in a *ppi2* background (T-DNA insertion, lacks atToc159). The M domain is sufficient to complement the mutation (although the transgenic plants only have 50% of the chlorophyll content of wild-type plants), showing that the GTPase function of Toc159 is partially dispensable *in vivo* (Lee, Kim et al. 2003). Wang *et al.* (2008) have expressed an atToc159 mutant with reduced GTPase activity in the *ppi2* background, showing full complementation. In fact, these mutant chloroplasts are able to import preproteins at a two-fold higher rate than wild-type chloroplasts, suggesting that atToc159 is locked into the active GTP bound state, increasing binding (Wang, Agne et al. 2008). The presence of non-hydrolyzable GTP analogues reduces preprotein binding and inhibits import, suggesting that Toc159 GTPase activity regulates the initial docking of preproteins to the translocon, while GTP hydrolysis by Toc34 results in the dissociation of preproteins from the receptors and transfer to the Toc channel (Wang, Agne et al. 2008). These data suggest that Toc159 functions as a receptor, not a motor; however, the order of association of preproteins with Toc159 and Toc34 remains to be elucidated.

1.6.1.3.2 Toc132/120/90

Toc159 has four homologues in *Arabidopsis thaliana*: atToc159, atToc132, atToc120, and atToc90, which show a high level of conservation within the G and M domains, but a high level of variation within the A domain, which accounts for the difference in molecular mass (absent in atToc90) (Hiltbrunner, Bauer et al. 2001). An *Arabidopsis thaliana* mutant line *ppi2* was created with a T-DNA insertion in the TOC159 gene, yielding albino plants containing no atToc159 mRNA or protein which do not survive past the cotyledon stage when grown on soil and contain undifferentiated proplastids (Bauer, Chen et al. 2000). This study has also shown the transcriptional repression of several normally highly abundant photosynthetic proteins in *ppi2* plants, even though the expression and import of non-photosynthetic chloroplast proteins was unaffected (Bauer, Chen et al. 2000). Smith *et al.* (2004) have shown that the *ppi2* phenotype is a result of the inability of these plants to import photosynthetic precursors, rather than a secondary effect of transcriptional repression. This study also showed the inability of non-photosynthetic plastid precursors to compete for binding to atToc159 with preFerredoxin, showing direct evidence of atToc159 selectivity for photosynthetic preproteins (Smith, Rounds et al. 2004). Hiltbrunner *et al.* (2004) have generated a knock out mutant for atToc90 without a visible phenotype; however, the atToc159 atToc90 double mutant plants showed more severely impaired chloroplast biogenesis than *ppi2* plants, without a defect in the import of several non-photosynthetic plastid preproteins. These results suggest that atToc159 and atToc90 may be involved in the import of photosynthetic preproteins, and that atToc132 and atToc120 may have a role in the import of non-photosynthetic plastid preproteins. Ivanova *et al.* (2004) have shown that these homologues form distinct complexes with atToc159 preferentially associated with atToc33 and atToc120 or atToc120/132 preferentially associated with atToc34. This study has also shown the preferential binding of atToc132 to a non-photosynthetic preprotein in an *in vitro* pull-down assay, which supports the hypothesis that atToc132/120 are involved

in the import of constitutive, non-photosynthetic preproteins (Ivanova, Smith et al. 2004). These data support the hypothesis that *Arabidopsis thaliana,* and possibly other species containing multiple isoforms of the Toc GTPases, contain multiple substrate specific translocons in outer plastid membranes to facilitate the import of highly expressed photosynthetic preproteins during photomorphogenesis as well as important, but less abundant, constitutive house-keeping proteins (Jarvis and Robinson 2004)

1.6.1.4 Toc64

A protein with the apparent molecular mass of 64 kDa has been identified by several studies to be a possible component of the Toc complex (Cornwell and Keegstra 1987; Hinz and Flugge 1988; Waegemann and Soll 1991). Sohrt and Soll (2000) fractionated digitonin solubilized outer envelope membranes from *P. sativum* over a sucrose density gradient, and a 64 kDa protein (Toc64) cofractionated with the Toc components Toc34, Toc75, and Toc86. A cDNA clone of this protein was generated, showing homology to amidases and indole acetamide hydrolases; however, due to a mutation of an essential active site residue (ser→gly), recombinant Toc64 failed enzymatic assays for amidase or amidotransferase activity. This study also showed that Toc64 behaves like an integral membrane protein and exposes a large cytosolic domain containing three tetratricopeptide (TPR) repeats (Sohrt and Soll 2000). TPRs contain a degenerate 34 amino acid repeated motif, are implicated in mediating protein-protein interactions, are found in a variety of organisms, subcellular locations, and processes, and have been identified in the mitochondrial import receptor complex (Tom70) and the peroxisomal import receptor complex (Lamb, Tugendreich et al. 1995). Schleiff *et al.* (2003) failed to observe Toc64 in the purified Toc complex, and suggested that Toc64 is only transiently associated with the other Toc components. Knockout mutants were generated in the moss *Physcomitrella patens* and in *Arabidopsis thaliana*; however, the mutants were indistinguishable from wild-type (Rosenbaum Hofmann and Theg 2005;

Aronsson, Boij et al. 2007). Qbadou *et al.,* (2006) have shown that the TPR domain of Toc64 interacts with preprotein complexed Hsp90 and that Toc64 hands off the preprotein to Toc34 in a nucleotide dependent manner, followed by the dissociation of Toc64 from the other Toc components. The experimental evidence suggests that Toc64 plays a role in the import of Hsp90 associated preproteins, but this function may be bypassed *in vivo*.

1.6.1.5 Toc12

Toc12 was identified by a proteome analysis of the outer envelope membrane of *P. sativum* (Becker, Hritz et al. 2004). This protein has a β-barrel-type anchor, and the C-terminus has high sequence similarity to the J-domain of DnaJ and projects into the intermembrane space. The J domain was shown to stimulate the ATPase activity of an Hsp70 homologue (Becker, Hritz et al. 2004). This subunit is postulated to recruit and activate an intermembrane space Hsp70, which will interact with the translocating preprotein and upon ATP hydrolysis, the preprotein will be transferred to the Tic complex. This component has only been identified in *P. sativum* to date (Kalanon and McFadden 2008).

1.6.2 The Organization of the Toc Hetero-oligomeric Complex

The main components of the Toc complex were identified over a decade ago using cross-linking and immunoprecipitation techniques isolating translocation complexes (Waegemann and Soll 1991; Hirsch, Muckel et al. 1994; Kessler, Blobel et al. 1994; Perry and Keegstra 1994; Schnell, Kessler et al. 1994; Wu, Seibert et al. 1994; Seedorf, Waegemann et al. 1995; Tranel, Froehlich et al. 1995), and most of the attention since that time has been spent on characterizing the functions of the various components (Jarvis 2008). Cross-linking studies are limited in that they do not necessarily allow the definition of the entire composition of an unknown protein complex due to chemical or conformational restrictions, i.e. only those components in close proximity to a trapped preprotein will be identified (Caliebe, Grimm et al. 1997). Soll and Waegemann (1992) have shown that it is possible to purify an intact, active Toc

complex by mild solubilization with the non-ionic detergent digitonin. This technique is not limited by the conformational restraints of cross-linking experiments, and allows for purification of complexes via immunoprecipitation. Label transfer cross-linking is a useful technique to identify and selectively label the translocon components that associate with importing preproteins, which has been used to map the sequential association of preprotein with the various Toc components during the import process (Perry and Keegstra 1994; Kouranov and Schnell 1997). This technique can be viewed as an affinity labeling procedure, and is particularly useful for the identification of new components, since the migration pattern of labeled proteins in SDS-PAGE is not altered by cross-linking preproteins to the complex components (Perry and Keegstra 1994). Ma *et al.* (1996) showed that Toc75 and Toc86 (proteolytic fragment of Toc159) were cross-linked to preprotein during the early stages of import, and also showed the association of Toc75, Toc86, and Toc34 by co-immunoprecipitation, illustrating the limitations of the former technique. During energy-independent binding, Toc75, Toc159, and Toc34 have been shown to be associated with preprotein via label transfer cross-linking; however, the interaction of the preprotein with Toc34 is nucleotide sensitive, as the inclusion of GMP-PNP abolished Toc34 labeling (Kouranov and Schnell 1997). The authors have proposed either a nucleotide dependence for the association of Toc34 with other Toc components, or a GTP induced conformational change in the Toc complex which is necessary for the insertion of the preprotein in the protein conducting channel of the outer membrane (Kouranov and Schnell 1997). The interaction of Tic110 and Hsp93 with the Toc complex even in the absence of preprotein was demonstrated by Nielson *et al.* (1997) by co-immunoprecipitation. This study also showed the importance of mild solubilization conditions, as the association of Hsp93 was only observed when non-ionic detergent was utilized. These studies have led to the identification of Toc components, but have not produced much information about the molecular architecture, organization, and dynamics of the Toc complex (Kikuchi, Hirohashi et al. 2006).

A stable, core Toc complex consisting of Toc75, Toc159, and Toc34 has been shown to exist in the outer chloroplast membrane even in the absence of preproteins (Waegemann and Soll 1991; Ma, Kouranov et al. 1996; Nielsen, Akita et al. 1997; Schleiff, Soll et al. 2003). Schleiff *et al.* (2003) have purified an active (binds preproteins in the presence of GTP), stable core complex of ~500 kDa that contains Toc159 (86 kDa fragment), Toc75, and Toc34 at a ratio of 1:4:4-6. The core complex was further characterized by transmission electron cryomicroscopy and low resolution three-dimensional reconstruction, indicating a dense outer ring, four inner pores, and a finger-like central projection (Schleiff, Soll et al. 2003). The authors speculate that this structure represents four Toc75 pores associated with four Toc34 receptors and a central Toc159, which correlates well with the measured stoichiometry (Schleiff, Soll et al. 2003). Kikuchi *et al.* (2006) calculated the molar ratio of the Toc components as 1:3:3 (Toc159:Toc75:Toc34) by quantification of the amounts of Toc75, Toc34, and Toc159 in isolated chloroplasts by standardized immunoblotting, with comparison to purified, recombinant Toc proteins and total chloroplast extracts.

1.6.3 Native Electrophoresis

In order to investigate the mitochondrial respiratory chain complex, (Schagger and von Jagow 1991; Schagger, Cramer et al. 1994) developed Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE), a novel electrophoresis technique for the isolation of active membrane protein complexes, which has a much higher resolution than sucrose density gradient centrifugation or gel filtration. Native membrane protein complexes can be isolated by mild solubilization with non-ionic detergents, and a negative charge is imposed by the binding of the dye Coomassie brilliant blue G-250 to hydrophobic domains on the surface of proteins, which also reduces the problem of protein aggregation (Schagger, Cramer et al. 1994). All membrane proteins and most soluble proteins bind Coomassie dye (Schagger, Cramer et al. 1994), inducing a negative charge, which allows for the separation of both acidic and basic proteins

at the fixed pH of 7.5, which is in the physiological range of most intracellular compartments (Krause 2006). Solubilization of membrane proteins with non-ionic detergents is often poor at low ionic strength; however, salts may lead to precipitation of Coomassie dye and stained protein, and should therefore be substituted with 6-aminocaproic acid (Schagger and von Jagow 1991; Schagger, Cramer et al. 1994). The solubilization conditions must be stringent enough to disrupt lipid-lipid interactions, while mild enough to preserve protein interactions within complexes as well as some structural lipid-protein interactions (Eubel, Braun et al. 2005). Therefore, only mild detergents such as *n*-Dodecyl maltoside, Triton X-100, and digitonin are suitable for the analysis of membrane protein complexes (Krause 2006). Two dimensional electrophoresis, with BN-PAGE in the first dimension and denaturing SDS-PAGE in the second dimension, is a high resolution method for the analysis of the molecular masses and oligomeric states of membrane protein complexes (Schagger, Cramer et al. 1994). The first dimension separates individual, native membrane protein complexes according to their molecular mass, followed by denaturation of protein complexes with SDS under reducing conditions. The lane is then placed on top of an SDS-PAGE gel and protein complexes are separated into their individual components according to molecular mass (Braun, Kinkl et al. 2007). This technique was developed to isolate native, active mitochondrial oxidative phosphorylation multiprotein complexes from bovine heart and yeast (Schagger and von Jagow 1991; Schagger, Cramer et al. 1994). BN-PAGE has since been utilized without major modification to examine the mitochondrial protein complexes of a large variety of eukaryotes, and has been extended to the analysis of the mitochondrial Tom and Tim translocons, the peroxisomal protein import machinery, thylakoid protein complexes, as well as protein complexes of the endoplasmic reticulum (for review, see (Krause 2006)). The Tic complex of *P. sativum* has been analyzed by BN-PAGE, leading to the identification of the putative Tic subunits Tic55 and Tic62 (Caliebe, Grimm et al. 1997; Kuchler, Decker et al. 2002). Kikuchi *et al.* (2006) were the first group to successfully apply this technique to the Toc

complex, most likely due to the complex's propensity for proteolytic degradation and/or difficulty in solubilizing isolated outer envelope membranes; therefore, intact *P. sativum* chloroplasts were solubilized and analyzed by BN-PAGE. This analysis revealed a Toc core complex composed of Toc75, Toc159, and Toc34, which has a molecular mass of 800-1000 kDa (Kikuchi, Hirohashi et al. 2006). The 86 kDa fragment of Toc159 was present in a complex at around 350-500 kDa with roughly half of Toc75 and Toc34 (Kikuchi, Hirohashi et al. 2006). Chen and Li (2007) cross-linked radiolabeled prSSU with the Toc complex during active import into intact chloroplasts, followed by purification by sucrose density gradient centrifugation, and analysis by BN-PAGE. The cross-linked Toc complex formed a two peak distribution in the sucrose gradient, with the larger peak corresponding with the Toc/Tic supercomplex and the smaller peak composed of at least two complexes migrating at ~880 kDa and ~1320 kDa. The smaller complex was composed of exclusively Toc components and correlates well with the complex characterized in the previously mentioned study, and the larger complex contained Toc components as well as Tic110, Hsp93, and an Hsp70 homologue, but not Tic40 and most likely represents a later stage in the import process (Chen and Li 2007).

1.6.4 Toc Complex Oligomeric Status & Dynamics

The small Toc GTPases are able to homodimerize (Sun, Forouhar et al. 2002; Weibel, Hiltbrunner et al. 2003; Reddick, Vaughn et al. 2007; Yeh, Kesavulu et al. 2007; Koenig, Oreb et al. 2008) and heterodimerize (Smith, Hiltbrunner et al. 2002; Jelic, Soll et al. 2003; Becker, Jelic et al. 2004) *in vitro*, possibly in a nucleotide dependent manner, although several studies provide seemingly contradictory results. Sun *et al.* (2002) published a crystal structure of the GDP loaded Toc34 dimer, and suggested the possibility of the homodimerization of Toc159 as well as the heterodimerization between the large and small Toc GTPases. Smith *et al.* (2002) confirmed the heterodimerization of the G domains of atToc33 and atToc159, stimulated by the inclusion of GDP in

comparison with GTP, ATP, and their nonhydrolyzable analogs. Jelic *et al.* (2003) have investigated the homo and heterodimerization of atToc33 and atToc34, and their results suggest that dimerization is unfavorable in the presence of GTP in comparison to GDP and no nucleotide, and heterodimerization is preferred to homodimerization. Koenig *et al.* (2008) have published crystal structures of psToc34 with GMP-PNP, atToc33 with GDP, and atToc33 with GMP-PNP, with very little structural rearrangement in the different nucleotide loaded states. This study has shown a slight preference for dimerization in the presence of GDP; however, the biochemical data suggest that the dimer is not a GAP complex due to the minimal increase in GTPase activity in the dimeric form (Koenig, Oreb et al. 2008). Becker *et al.* (2004) have investigated the GDP or GMP-PNP dependence of the association of Toc components by immunoprecipitation of solubilized outer envelope vesicles, as well as by affinity chromatography, which allows for the differential nucleotide loading state of the two GTPases. All three core components were coimmunoprecipitated with similar efficiency in the presence of GMP-PNP or in the absence of nucleotides by Toc34 or Toc159 antisera. GDP incubation seems to destabilize the complex, which leads to the reduced coimmunoprecipitation of Toc34 with Toc159 antiserum and *vice versa*. The affinity chromatography results suggest that heterodimerization is favored in the GMP-PNP bound state, and is stabilized by the presence of transit peptide (Becker, Jelic et al. 2004). The amount of Toc75 immunoprecipitated by Toc34 antiserum in the presence of GDP was decreased, whereas the association of Toc75 and Toc159 seems to be nucleotide independent (Becker, Jelic et al. 2004). The association between Toc34 and Toc75 is mediated by a cytosolic loop in the N-terminal region of Toc75 (Ertel, Mirus et al. 2005). The highly dynamic nature of the Toc complex is illustrated by the influence of energetics on preprotein association with Toc components (Kouranov and Schnell 1997; Young, Keegstra et al. 1999) as well as the interactions between Toc components (Becker, Jelic et al. 2004), the different ratios of Toc components in the isolated complex and in the membrane

(1:1 for Toc75:Toc34 in the isolated complex compared to 4:1 in the outer envelope *in situ*) (Schleiff, Soll et al. 2003), the multiple size species observed from BN-PAGE (Kikuchi, Hirohashi et al. 2006), and the differential ability of the Toc GTPases (soluble domains) to participate in homodimeric interactions (Sun, Forouhar et al. 2002; Weibel, Hiltbrunner et al. 2003; Reddick, Vaughn et al. 2007; Yeh, Kesavulu et al. 2007; Koenig, Oreb et al. 2008), heterodimeric interactions both between atToc33 and atToc34 (Jelic, Soll et al. 2003) and between the large and small GTPases (Smith, Hiltbrunner et al. 2002; Becker, Jelic et al. 2004).

1.7 The Tic Apparatus

As the transit peptide of a preprotein emerges from the Toc complex, it is engaged by the Tic complex and translocated across the inner envelope membrane. The transit peptide is cleaved as it emerges into the stroma, before translocation is completed (Schnell and Blobel 1993). Most translocation probably occurs simultaneously through the Toc and Tic complexes at contact sites, but these complexes can function independently under certain conditions *in vitro* (Scott and Theg 1996). The motor function associated with the Tic complex has been proposed to be carried out by stromal Hsp93 (Jackson-Constan, Akita et al. 2001), which agrees with the stromal requirement for ATP for translocation to occur (Theg, Bauerle et al. 1989).

Components of the Tic (translocon at the inner chloroplast envelope membrane) complex have been identified by covalent cross-linking; however, their functions have not been characterized as well as those of the Toc complex (Jarvis 2008). The main components of the Tic complex include Tic110, Tic40, Tic22, and Tic20 (Smith 2006). The Tic components Tic55, Tic62, and Tic32 have been implicated in the redox regulation of protein translocation (Caliebe, Grimm et al. 1997; Kuchler, Decker et al. 2002; Hormann, Kuchler et al. 2004) for Review see (Bedard and Jarvis 2005).

1.7.1 The Tic Subunits

1.7.1.1 Tic110

Tic110 is a major component in the inner chloroplast envelope membrane (Cline, Andrews et al. 1981; Kessler and Blobel 1996), and has been identified as a member of the Tic complex by cross-linking and co-immunoprecipitation studies (Lubeck, Soll et al. 1996). Two different models have been proposed to describe the topology of Tic110; however, both models agree on the presence of two hydrophobic N-terminal transmembrane α-helices, and the presence of a large stromal domain (Balsera, Goetze et al. 2008). One model proposes that most of Tic110 is composed of a large (>90 kDa), stromally located hydrophilic domain (Jackson, Froehlich et al. 1998). The second model proposes that Tic110 functions as the Tic channel, and is composed of four amphipathic transmembrane helices and two large intermembrane space regions (Balsera, Goetze et al. 2008). In the presence of preproteins, Tic110 interacts with the Tic components Tic40, Tic22, Tic20 (Wu, Seibert et al. 1994; Kouranov, Chen et al. 1998), the soluble, stromal chaperones Hsp93 and Cpn60 (Kessler and Blobel 1996; Chou, Fitzpatrick et al. 2003), as well as the Toc components Toc159, Toc34, and Toc75 (Akita, Nielsen et al. 1997; Nielsen, Akita et al. 1997) forming the Toc/Tic supercomplex. atTic110 functions as a scaffold for the recruitment of stromal chaperones, contains a preprotein binding site adjacent to the exit site of the Tic translocon, and forms the backbone of the Tic import motor complex (Inaba, Li et al. 2003). atTic110 null mutants are embryo lethal, and reduction in expression leads to defects in plastid biogenesis (Inaba, Li et al. 2003); however, none of the combinations of Tic110, Tic40, and Hsp93 double mutants show additivity, suggesting a close functional relationship of the components (Kovacheva, Bedard et al. 2005). The controversy surrounding the orientation of this protein has not yet been resolved, but the association of Tic110 with Toc components and intermembrane space-located Tic22 as well as with stromal Tic components and chaperones seems to support the second model.

1.7.1.2 Tic40

Tic40 is an integral membrane protein of the chloroplast inner envelope membrane with a large hydrophilic domain projected into the stroma (Chou, Fitzpatrick et al. 2003). Tic40 has been shown to function with Tic110 and Hsp93 at a late stage of protein import (Chou, Fitzpatrick et al. 2003; Kovacheva, Bedard et al. 2005). *Arabidopsis tic40* mutant plants are not seedling lethal (Chou, Fitzpatrick et al. 2003), and the expression of atTic40 deletion constructs yields a more severe phenotype than the null mutants (Bedard, Kubis et al. 2007), suggesting a stimulatory/regulatory role for Tic40. The stromal domain of Tic40 contains a tetratricopeptide (TPR) protein interaction domain followed by a domain with significant homology to mammalian Hip (Hsp70 interacting protein) and Hop (Hsp70 and Hsp90 organizing protein, aka p60 or Sti1p) co-chaperones (Chou, Fitzpatrick et al. 2003). Hip interacts with Hsp70, regulating its ATPase cycle by stabilizing the ADP bound, high substrate affinity form, and may contribute to the interaction of Hsp70 with target proteins by its own chaperone activity (Hohfeld, Minami et al. 1995). Tic40 does not bind preproteins directly, but has been shown to initiate the release of transit peptides from Tic110 and to stimulate Hsp93 ATP hydrolysis (Chou, Chu et al. 2006). A model has been proposed by Chou *et al.* (2006) in which the transit peptide binds to Tic110 as it emerges from the Tic channel, recruiting binding of the Tic40 TPR domain to Tic110, stimulating transit peptide release and cleavage by SPP. The Hip/Hop domain of Tic40 then stimulates Hsp93 ATP hydrolysis, providing the driving force for protein translocation across the inner envelope membrane (Chou, Chu et al. 2006).

1.7.1.3 Tic20 and Tic22

Tic20 and Tic22 have been identified as major targets of cross-linking during the late stages of preprotein import (Ma, Kouranov et al. 1996; Kouranov and Schnell 1997). Tic22 is located in the intermembrane space, peripherally

associated with the inner membrane, and is most likely the first Tic component to be encountered by a translocating preprotein (Kouranov, Chen et al. 1998). Tic22 most likely functions in directing preproteins from Toc to Tic, may participate with the J Domain containing Toc12 and an Hsp70 in the intermembrane space, and might function in the formation of Toc/Tic supercomplexes (Jarvis 2008). Tic20 is an integral membrane protein containing four predicted α-helical transmembrane segments and small N and C terminal soluble domains (Kouranov, Chen et al. 1998). Tic20 shows some sequence similarity with bacterial branched chain amino acid transporters (Reumann and Keegstra 1999), which along with the topological and cross-linking data, suggests that Tic20 may form part of the translocon channel of the inner membrane.

1.8 Cytosolic Factors and Chaperones

Like the transport systems of the endoplasmic reticulum and mitochondria, transport of preproteins from the cytosol into chloroplasts relies on the action of molecular chaperones, which are the most likely candidates for the location of ATP hydrolysis (Jackson-Constan, Akita et al. 2001), which has been shown to be necessary for translocation to occur (Theg, Bauerle et al. 1989; Olsen and Keegstra 1992). These chaperones include cytoplasmic, envelope-associated, and stromal Hsp70 chaperones, stromal Hsp100 and Hsp60 chaperones, and cytoplasmic 14-3-3 proteins (Jackson-Constan, Akita et al. 2001). The transit peptides of major chloroplast proteins can be phosphorylated by a cytoplasmic protein kinase; however, these precursors must be dephosphorylated before translocation can occur, although phosphorylation does not interfere with binding (Waegemann and Soll 1996). Phosphorylated preproteins have been shown to interact with a ‘guidance complex’ containing 14-3-3 proteins and Hsp70, requiring ATP for complex dissociation, which has been shown to increase import efficiency *in vitro* (May and Soll 2000). Some transit peptides do not contain a 14-3-3 binding motif, and mutagenesis resulting in the removal of this

phosphorylation site does not prevent targeting to the chloroplast *in vivo,* suggesting that this guidance complex may function in increasing the import efficiency of highly expressed chloroplast proteins (Nakrieko, Mould et al. 2004). Qbadou *et al.* (2006) have identified an alternative pathway in which Hsp90 complexed preproteins are recognized by the TPR domain of Toc64, which mediates preprotein transfer to Toc34 in a nucleotide dependent fashion. Hsp70 proteins interact with nascent chains to prevent aggregation and misfolding, and their interaction with integral membrane proteins synthesized in the cytosol, destined for the chloroplast may be especially important (Jackson-Constan, Akita et al. 2001). Hsp70 chaperones contain two functional domains: an ATP binding domain and a substrate binding domain, and preferentially bind to hydrophobic and positively charged amino acids, but not to acidic residues (Zhang and Glaser 2002). Greater than 75% of the transit peptides in the CHLPEP database contain a putative Hsp70 recognition site in the N-terminal third, and the majority of transit peptides contain a second Hsp70 recognition site, with enough separation to allow the simultaneous engagement of Hsp70s on either side of the bilayer, which could function to prevent retrograde movement (Ivey, Subramanian et al. 2000). Two Hsp70 proteins are associated with the outer envelope membrane: Com70 is associated with the cytoplasmic side of the outer envelope membrane (Ko, Bornemisza et al. 1992) and Hsp70-import associated protein (IAP) is located in the intermembrane space (Schnell, Kessler et al. 1994). Translocation can occur across each envelope membrane independently (Scott and Theg 1996), and Hsp70-IAP is the best candidate for the outer envelope membrane translocation motor (Jackson-Constan, Akita et al. 2001), and is most likely stimulated by the J-domain of Toc12 (Becker, Hritz et al. 2004). The Hsp100 homologue Hsp93 (formerly known as ClpC) functions in the chloroplast stroma to bind emerging preproteins, prevent retrograde movement, and to act as the stromal translocation motor (Jackson-Constan, Akita et al. 2001). The interaction between Tic110 and Tic40 has been well established (Stahl, Glockmann et al. 1999; Chou, Chu et al. 2006; Bedard, Kubis et al. 2007),

and Hsp93 has also been shown to interact with these two Tic components (Chou, Fitzpatrick et al. 2003). Chou *et al.* (2006) have proposed the following model for preprotein translocation into the stroma: the emerging preprotein is bound by the N-terminal portion of the Tic110 stromal domain causing a conformational change which recruits the binding of the Tic40 TPR domain to Tic110, causing transit peptide release and cleavage by the stromal processing peptidase (SPP). The binding of the Tic40 TPR domain to Tic110 unshields the Tic40 Hip domain, which stimulates ATP hydrolysis by Hsp93, driving protein import into the stroma (Chou, Chu et al. 2006). Newly imported proteins most likely interact with stromal Hsp70s, Cpn60 (GroEL homologue), and 14-3-3 proteins, as well as their co-chaperones, in order to mediate proper folding or to maintain thylakoid preproteins in an transport competent state through the stroma (Jackson-Constan, Akita et al. 2001; Jarvis 2008).

1.9 Lipids of the Outer Envelope Membrane

Like their cyanobacterial ancestors, chloroplast membranes are characterized by a large amount of glycolipids and a low content of phospholipids, with phosphatidylcholine (PC) as the major outer envelope phospholipid, and phosphatidylglycerol (PG) as the major chloroplast phospholipid (Joyard, Block et al. 1991). The glycolipids present in the chloroplast membranes are sulfolipid (SL), which carries a negative charge, and the major chloroplast glycolipids are the neutral galactolipids monogalactosyldiacylglyceride (MGDG) and digalactosyldiacylglyceride (DGDG) (Joyard, Block et al. 1991). The chloroplast outer envelope is the only cytosolically exposed plant membrane containing MGDG and DGDG (Bruce, 1998). The chloroplast outer envelope membrane has a very high lipid to protein ratio (2.5-3.0 mg lipids/mg protein), which is 3 times higher than the inner envelope, and 6-8 times higher than the thylakoids (Block, Dorne et al. 1983). The transit peptides of ferredoxin and the small subunit of rubisco have been shown to interact specifically with outer envelope lipids (van't Hof, van

Klompenburg et al. 1993; Pilon, Wienk et al. 1995; Pinnaduwage and Bruce 1996), and this interaction may lead to the induction of secondary structural elements (i.e. α-helices) in the transit peptides (Horniak, Pilon et al. 1993; Wienk, Czisch et al. 1999; Wienk, Wechselberger et al. 2000).

1.10 Proposed Research

My thesis proposes using the relatively rapid and highly sensitive nature of Blue-Native Polyacrylamide Gel Electrophoresis coupled with western blotting to explore the size and distribution of the Toc subunits. The influence of nucleotides on the interaction between soluble domains of Toc components has been investigated, with some contradictory results; however, the relevance of these interactions to the *in vivo* state in the membrane can be questioned due to the absence of additional domains (membrane anchors and acidic domains), lipids, and interacting partners (i.e. Toc75). Previously, two groups have characterized the Toc complex using BN-PAGE and provided insight into the size and compostion of the core Toc complex (Kikuchi, Hirohashi et al. 2006) as well as the Toc complex and associated proteins cross-linked, and isolated under active import conditions (Chen and Li 2007). I will use two dimensional electrophoresis (BN-PAGE in the first and SDS-PAGE in the second dimension) to define the components of the hetero-oligomeric Toc complex. Using this system, I will investigate the influence of experimental conditions on complex dynamics and induced oligomerization or dissociation of components. The dynamics of the interactions between the Toc components in their native membrane will be investigated by incubating intact chloroplasts with various nucleotides to modulate the energetic state of the translocon (ATP, GDP, GTP, non-hydrolyzable GMP-PNP and XTP, a transition state analog, preprotein, and isolated soluble domains of Toc subunits). The aims of this research are: 1) to investigate developmental changes in the composition of the chloroplast import apparatus, 2) nucleotide-dependent oligomeric changes, 3) size shift induced by incubation of chloroplasts with preprotein in binding and active import conditions,

and 4) to investigate the possible structural function of N-terminal POTRA domains of Toc75 in Toc complex assembly.

CHAPTER II

2.0 MATERIALS AND METHODS

2.1 Plant Growth and Chloroplast Isolation

Chloroplasts were isolated from 12-14 day old Progress #9 pea (*Pisum sativum*) seedlings. Approximately 350 ml dry seeds (Jung Seed, Randolph WI) were imbibed overnight with aeration per flat. Hydrated seeds were then planted in a 12x24x5 inch metal flat with A-3 horticultural vermiculite (Knoxville Seed and Greenhouse). Pea seedlings were grown in an EGC growth chamber with incandescent and fluorescent illumination at 160 μE/m^2/sec on a 14 hour light and 10 hour dark cycle.

Approximately 30 g of pea tissue was harvested with scissors after ten hours of dark conditioning to minimize starch accumulation. The tissue was chopped in a Cuisinart food processor with 3 to 5 short pulses (1 to 2 sec) before transfer to an ice cold mortar. All of the following steps were carried out at 4ºC in the dark or under dim light with 5 μl/ml (unless otherwise stated) protease inhibitor cocktail (PIC) for plant cell and tissue extracts (Sigma P-9599). To the chopped pea tissue, 30 ml of ice cold Grinding Buffer (GB: 50 mM HEPES-KOH pH 7.3, 330 mM Sorbitol, 1 mM $MgCl_2$, 2 mM EDTA, 0.1% BSA, 10 μl/ml PIC) with 0.3g of fresh sodium ascorbate was added, and the tissue was homogenized with pestle. Homogenate was then filtered through one layer of cheese cloth topped with one layer of Miracloth into a 50 ml tube on ice. Chloroplasts were pelleted by centrifugation at 1500 g for 3 min, and the supernatant was decanted. The chloroplasts were then resuspended in 4 ml of Import Buffer (IB: 50 mM HEPES-KOH pH 8.0, 330 mM Sorbitol) with a natural bristle paintbrush, by gently painting the chloroplasts up the side of the tube. The resuspended chloroplasts were then loaded onto a previously prepared Percoll step gradient in a 15 ml tube (4 ml of 40% Percoll in IB overlaid on 2 ml of 80% Percoll in IB) with a 14 gauge stainless steel cannula attached to a 10 ml syringe. The intact chloroplasts were separated from the broken chloroplasts by

centrifugation at 3500g for 10 min with acceleration and deceleration set to medium. The intact chloroplasts were then carefully harvested from the 40/80 interface with syringe taking care to minimize shear forces, leaving behind broken chloroplasts and thylakoids on top of the 40% Percoll. With the same syringe, 4 ml of IB were pulled up and the syringe was inverted several times to resuspend chloroplasts. The intact chloroplasts were then transferred to a new 15 ml tube and pelleted by centrifugation at 1500 g for 3 min, and the supernatant was decanted.

2.2 TIRF Microscopy

Total Internal Reflection Fluorescent (TIRF) microscopy is a technique that allows for the excitation of fluorophores near a solid surface without exciting more distal fluorophores. The total internal reflection of the excitation laser beam yields an evanescent field which excites fluorophores within 100 nm of the surface, leading to low background fluorescence and a high signal to noise ratio (Axelrod 2001). With TIRF microscopy, single fluorescent molecules can be observed, and their dynamics and kinetics can be studied, while minimizing photobleaching and photodamage (Toomre and Manstein 2001).

In order to visualize the dynamic environment of the Toc complex, Toc159 was labeled with a fluorescent antibody, and was observed with a TIRF microscope in order to minimize the influence of chlorophyll autofluorescence. Purified, intact chloroplasts were resuspended in 200 µl IB with 1% BSA in a 1.5 ml tube and blocked for 30 min with rocking (all steps take place at 4ºC). Next, the chloroplasts were labeled with Toc159 1º antibody (courtesy of Danny Schnell) for 30 min with rocking at a titer of 1:500. The chloroplasts were then washed by carefully underlaying 500 µl of 40% Percoll in IB with a glass Pasteur pipet and centrifuging at 3500 g for 8 min. The supernatant was removed, chloroplasts resuspended in IB, and the wash was repeated. The chloroplasts were then resuspended in IB with 1% BSA and incubated with Alexa 488 conjugated 2º antibody for 30 min with rocking at a titer of 1:500. Chloroplasts

were then washed two times as before. After resuspension with 200 µl of IB, chloroplasts were further diluted with IB and visualized with a Nikon Inverted Laser TIRF microscope. If labeled chloroplast solution was too concentrated, the sample was removed and further diluted with IB. Video files were generated and particle tracking was performed with NIS-Elements software package.

2.3 Chlorophyll Measurement

The concentrations of chlorophyll a and b were determined by extraction into 80% acetone, and spectrophotometric analysis at 663 and 645 nm. 50 µl of chloroplast suspension was added to 5 ml 80% acetone and centrifuged at 14,000 x g for 3 min to remove starch. Absorbance measurements were taken at 663 and 645 nm and the following equation was used to calculate chlorophyll concentration (Arnon 1949).

$$\text{Chlorophyll (mg/ml)} = \frac{((8.02 \times A_{663}) + (20.2 \times A_{645}))}{0.05 \text{ ml} \times 1000 \text{ µg/mg}} \times 5 \text{ ml}$$

2.4 Envelope Preparation

In order to purify crude envelopes (combined inner and outer chloroplast membranes), purified chloroplasts were mixed with 12.5 mM MOPS-NaOH (pH 7.0), 2.5 mM magnesium acetate to yield a final chlorophyll concentration of 1.5 mg /ml and lysis was continued on ice in the dark for 10 min. The lysate was centrifuged at 1500 g for 5 min. to pellet thylakoids and the green supernatant was removed and stored on ice. The remaining material was centrifuged at 4000 g for 5 min and the yellow supernatant fraction was combined with the previously removed fraction. The combined envelopes were pelleted by centrifugation at 48,000 g for 30 min (adapted from (Markwell, Bruce et al. 1992)).

2.5 Native PAGE

Intact chloroplasts are resuspended in 1 ml IB and divided equally into 3 1.5 ml tubes. 330 µl of the perturbant (nucleotide/peptide/protein) solution to be

tested for change in oligomeric state of Toc complex was then added to the tube corresponding to the longest time point to be tested (30 min), the solution was mixed with a pipette, and the tubes were incubated in the dark at 25ºC. After 15 min., 330 µl of the experimental solution was added to the tube corresponding to the second time point, 330 µl of IB was added to the control tube, the tubes were mixed by pipette, and incubation was continued for an additional 15 min in the dark.

At the end of the incubation, the unbound perturbant and broken chloroplasts were removed from the intact chloroplasts by overlaying the solution on 500 µl 40% Percoll in IB and centrifuging at 3500 g for 8 min. in a swinging bucket rotor. The supernatants were decanted, and the chloroplast pellets were resuspended in 175 µl Solubilization Buffer (SB: 1% digitonin in 50 mM BisTris-methane pH 7.0, 500 mM aminocaproic acid, 10% glycerol) at ~0.5 mg chlorophyll/ml and incubated for 10 min. on ice in the dark. Insoluble material was removed by ultracentrifugation at 100,000 g for 10 min. The supernatants were removed and mixed with Coomassie G-250 solution (5% Brilliant Blue G-250, 50 mM BisTris pH 7.0, 500 mM aminocaproic acid) to yield an 8:1 detergent to Coomassie ratio.

The solubilized chloroplast samples were loaded in triplicate on a 0.75 mm thick 4-10% polyacrylamide gradient gel containing 50 mM BisTris and 500 mM aminocaproic acid and run for 5 hours at 200 V at 4ºC. BSA (66 and 132 kDa) and Ferritin (440, 880, and 1320 kDa) were used as molecular weight markers. The anode buffer was 50 mM BisTris pH 7.0. The initial cathode buffer was 50 mM Tricine, 15 mM BisTris pH 7.0, and 0.02% Brilliant Blue G-250, and after 1.5 hour was exchanged for cathode buffer of the same composition without G-250 dye. At the conclusion of the gel run, the lane containing the molecular weight markers was separated and stained. The other individual lanes were separated and heated at 65ºC in buffer containing 3.3% SDS, 4% βME, and 65 mM Tris-HCl pH 6.8 for 20 min.

2.6 2d SDS-PAGE and Western Blotting

Six 1.5 mm 8-16% SDS-PAGE gradient mini-gels were poured, overlaid with 50% isopropanol, and allowed to polymerize. The isopropanol was removed, and approximately 0.75 ml of 4.8% stacking gel was poured on the resolving gels, topped with 50% isopropanol, and allowed to polymerize. The isopropanol was removed, and a lane from the first dimension native gel was rinsed in SDS-PAGE running buffer and placed horizontally on top of the stacking gel along with a 1 well comb for molecular weight standards. The lane was sealed in and the well was created with 4.8% stacking gel. This process was repeated 5 times, yielding two gels for each experimental time point as well as two gels for the control. The gels were run at ~30 mA per gel for about 1 hour until the dye front ran off.

The gels were transferred to Immobilon PVDF with a Genie electrophoretic transfer apparatus (Idea Scientific) for 4 hours at 4ºC. The transfer buffer used depended on the protein to be analyzed: For western blotting against Toc75 and Toc159, proteins were transferred in high molecular weight transfer buffer (48 mM Tris, 390 mM Glycine, 0.1% SDS, 20 % methanol). For blotting against Toc34, transfer was accomplished in high pH transfer buffer (100 mM Tris, 192 mM Glycine, 20% methanol).

The Immobilon was blocked with TBST (25 mM Tris-HCl pH 8.0, 137 mM NaCl, 3 mM KCl, 0.1% Tween 20) containing 3% nonfat milk and 0.5% BSA for 1 hour at room temperature with rocking. The blots were incubated with the 1º antibodies for 2 hours at a 1:25,000 titer in blocking buffer. The blots were then washed 3 times with TBST for 10 min. The blots were incubated with the 2º antibodies for 1 hour at a 1:25,000 titer in blocking buffer. The blots were again washed 3 times with TBST for 10 min. The blots were incubated with 1.5 ml HRP chemiluminescent substrate per blot and photon counting was performed on a Chemidoc system (BioRad). The blots were analyzed with Quantity One software (BioRad) and the complexes formed at different sizes were quantitated. In order to reprobe a blot with a different antibody, the PVDF was stripped by

incubation in stripping solution (25 mM glycine-HCl pH 2.0, 1% (w/v) SDS) for 30 minutes with rocking at room temperature (See Immobilon Blotting Protocols). Blots were washed for 10 min x 2 with TBST, then next western blot protocol began with blocking the membrane.

CHAPTER III
3.0 2D ELECTROPHORESIS OF TOC COMPLEX

3.1 Introduction

Plastids only originate from pre-existing plastids, and chloroplasts in photosynthetic leaf cells generally develop from meristematic proplastids in a differentiation process that is regulated by light (Lopez-Juez and Pyke 2005). During this process, chloroplasts accumulate thylakoid membrane proteins involved in the light reactions of photosynthesis and soluble proteins that participate in CO_2 fixation (Mullet 1988). Dahlin and Cline (1991) have shown that import capability is correlated with plastid development, and declined as much as 20-fold as chloroplasts reached maturity. The Toc complex, responsible for the translocation of preproteins transcribed in the cytosol across the outer envelope membrane of the chloroplast, is composed of Toc75, Toc159, and Toc34 as well as several other components which may be dynamically associated. Fulgosi et al. (2005) have shown that Toc34 isoforms differentially accumulate during spruce needle development and Toc159 levels are drastically reduced during chloroplast morphogenesis. It is therefore interesting to speculate on the presence of Toc complexes of differing sizes and compositions as chloroplasts mature, and their protein import needs change.

The Toc complex is a dynamic translocon, which is composed of three core components, but several other components and chaperones are dynamically associated. The homo and heterodimerization of the isolated soluble domains of the Toc GTPases is affected by the nucleotide loading state of the proteins, and at least one study has implicated an effect of the nucleotide state on complex stability. The affinity of Toc34 for preproteins has been shown to be modulated by nucleotides, and GTP hydrolysis at Toc34 is necessary for import. The GDP:AlF_4 complex has been used in the structural analysis of GTPases as a transition state analog (Focia, Gawronski-Salerno et al. 2006), due to the occupation of a site near that occupied by the γ-phosphate of GTP and the aluminum fluoride is stabilized by interacting with residues that are

essential for GTPase activity (Sondek, Lambright et al. 1994), and therefore may give insight into the structural significance of GTP hydrolysis on Toc complex stability. During active import, preproteins are usually translocated across both envelope membranes through the coupled actions of the Toc/Tic supercomplex, and Tic components have been shown to associate with the Toc complex even in the absence of preproteins. Akita et al. (1997) analyzed cross-linked complexes arrested in the the presence of 75 μM ATP on denaturing SDS-PAGE, and observed three complexes containing Toc75, Toc34, and the 86 kDa fragment of Toc159: the largest was not able to enter the gel and contained at least the Toc components, Tic110, and Hsp93. The second complex had an estimated molecular mass of 500-700 kDa, and the heavier portion of the complex contained all five proteins; the last complex only contained the Toc components and had an estimated molecular mass of 250-350 kDa (Akita, Nielsen et al. 1997). The size of the core Toc complex has been estimated at ~500 kDa (Schleiff, Soll et al. 2003) and 880-1000 kDa (Kikuchi, Hirohashi et al. 2006), and when crosslinked and isolated during active import, the size has been estimated at 1320 containing the Toc components, Tic110, Hsp93, an hsp70 homologue, but not Tic40, and even higher for the supercomplex (Chen and Li 2007).

The stoichiometry of the core Toc components has been estimated as 1:4:4-6 (Schleiff, Soll et al. 2003) and 1:3:3 (Kikuchi, Hirohashi et al. 2006) (Toc159:Toc75:Toc34), which would correspond to a size range of 413-663 kDa for the various ratios and possible degradation of Toc159 to the 86 kDa fragment. Therefore, these ratios suggest that the larger complexes that have been observed are composed of a superdimer of the core Toc components, and possibly other unidentified accessory proteins. The Toc complex will be characterized by 2d electrophoresis, and the presence of Toc components will be compared in the size ranges corresponding to the Toc/Tic supercomplex, the range corresponding to a superdimer of Toc components which may be associated with Tic110 and Hsp93 (1400-880 kDa), an intermediate size range corresponding to the largest cross-linked complex analyzed by Akita et al. (1997)

containing the three Toc components, Tic110, and Hsp93 (880-630 kDa), and the range of smaller Toc complexes such as the complex Schleif et al. (2003) characterized by cryo EM and the smaller complexes observed by Kikuchi et al. (2006) after limited proteolytic degradation (630- ~200 kDa).

3.2 Results

In order to examine the influence of experimental conditions on the distribution of the Toc components in 2d PAGE, the amount of variability inherent to the assay must first be determined. Therefore, chloroplasts were isolated, solubilized, and analyzed with BN-PAGE. The lanes were treated with SDS under reducing conditions, and individual proteins were separated by 2d SDS-PAGE. The 2d gels were then transferred to Immobilon PVDF, and western blot hybridization was performed in triplicate with α-Toc159 antiserum. The blots are shown in Fig. 3-1 A, C, & E, and the quantitation of these blots is shown in Fig. 3-2. This procedure was repeated with a separate chloroplast prep, and the blots are shown in Fig. 3-1 B, D, & F and the quantitation is shown in Fig. 3-2 and Table 3-1. One set of these blots was stripped by the low pH and detergent method (Millipore) and re-probed with α-Toc75 antiserum, and the blots are shown in Fig 3-4 A, C, & E and the quantitation of these blots is shown in Fig. 3-4 G & H. The mean and standard deviation was calculated for each of the size ranges. Intact Toc159 migrates somewhat aberrantly during electrophoresis, with an apparent size of 200-230 kDa in SDS-PAGE (Bolter, May et al. 1998; Hiltbrunner, Bauer et al. 2001). The standard deviation was minimized both within one prep and between two preps by combining the amounts of intact Toc159 participating in the Toc/Tic supercomplex and in the 1400-630 kDa range; therefore, all experimental conditions will compare the total amount of intact Toc159 with the amount of the 86 kDa fragment (Toc159f) in the four size ranges: supercomplex, 1400-880 kDa, 880-630 kDa, and 630-200 kDa.

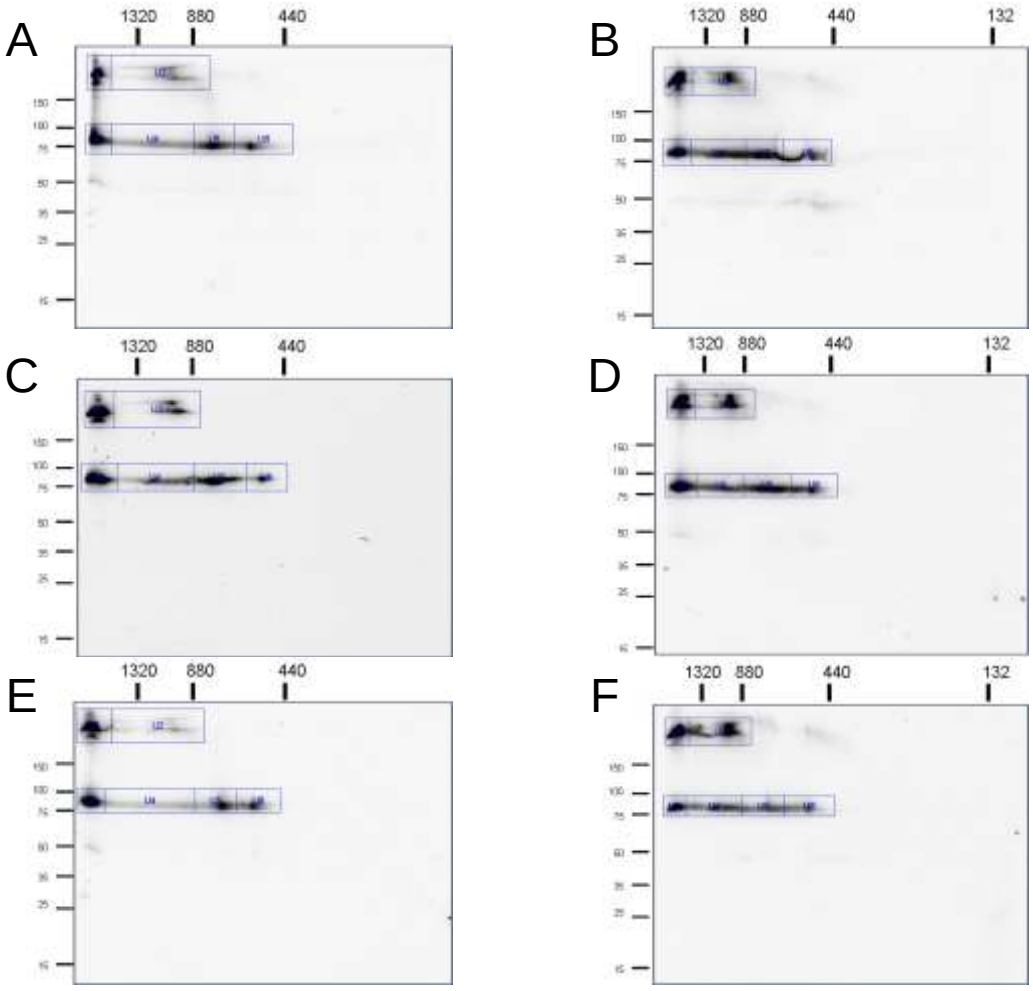


Figure 3-1 Control experiment for two-dimensional BN/SDS-PAGE analysis of Toc159.

A, C, & E are three replicates of western blots from 2d gels of control 1d lanes from the first chloroplast prep. B, D, & F are three control replicates from a separate chloroplast prep and 1d gel.

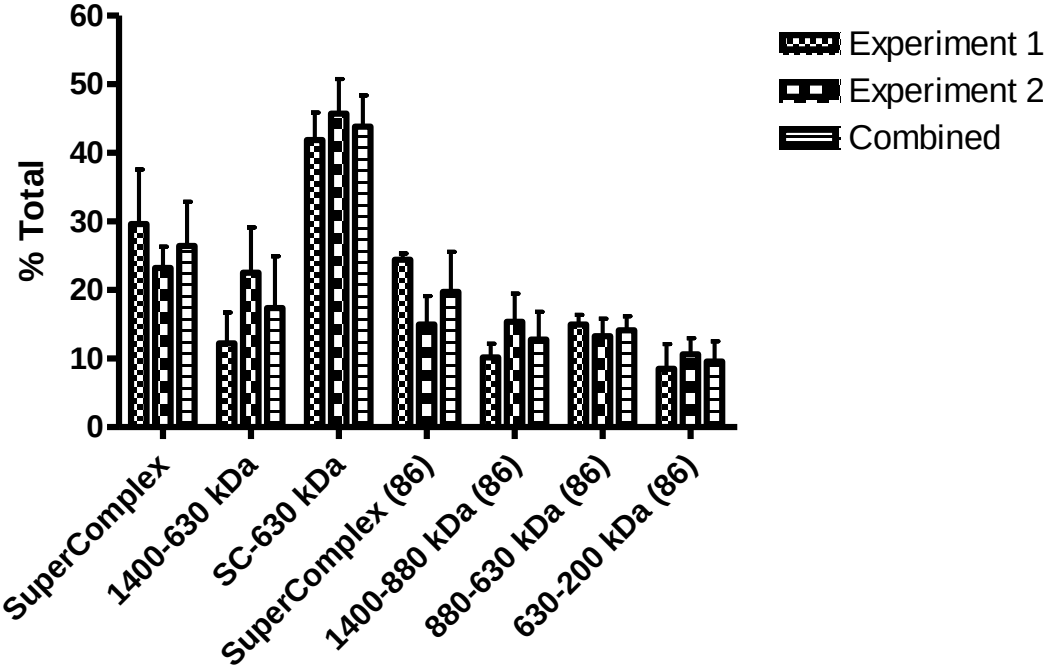


Figure 3-2 Control experiment for two-dimensional BN/SDS-PAGE analysis of Toc159.
The first and second sets of bars represent the data from the two control experiments separately, and the third set is the combined data.

Table 3-1 Control experiment for two-dimensional BN/SDS-PAGE analysis of Toc159.

	Experiment 1		Experiment 2		Combined	
	Average	StDev	Average	StDev	Average	StDev
SuperComplex	29.7	7.93	23.2	3.09	26.4	6.43
1400-630 kDa	12.2	4.48	22.5	6.59	17.4	7.56
SC-630 kDa	41.9	4	45.7	5.03	43.8	4.59
SuperComplex (86)	24.5	0.88	15.0	4.14	19.7	5.84
1400-880 kDa (86)	10.2	1.99	15.4	4.1	12.8	4.05
880-630 kDa (86)	14.9	1.42	13.3	2.55	14.1	2.06
630-200 kDa (86)	8.5	3.61	10.6	2.37	9.6	2.96

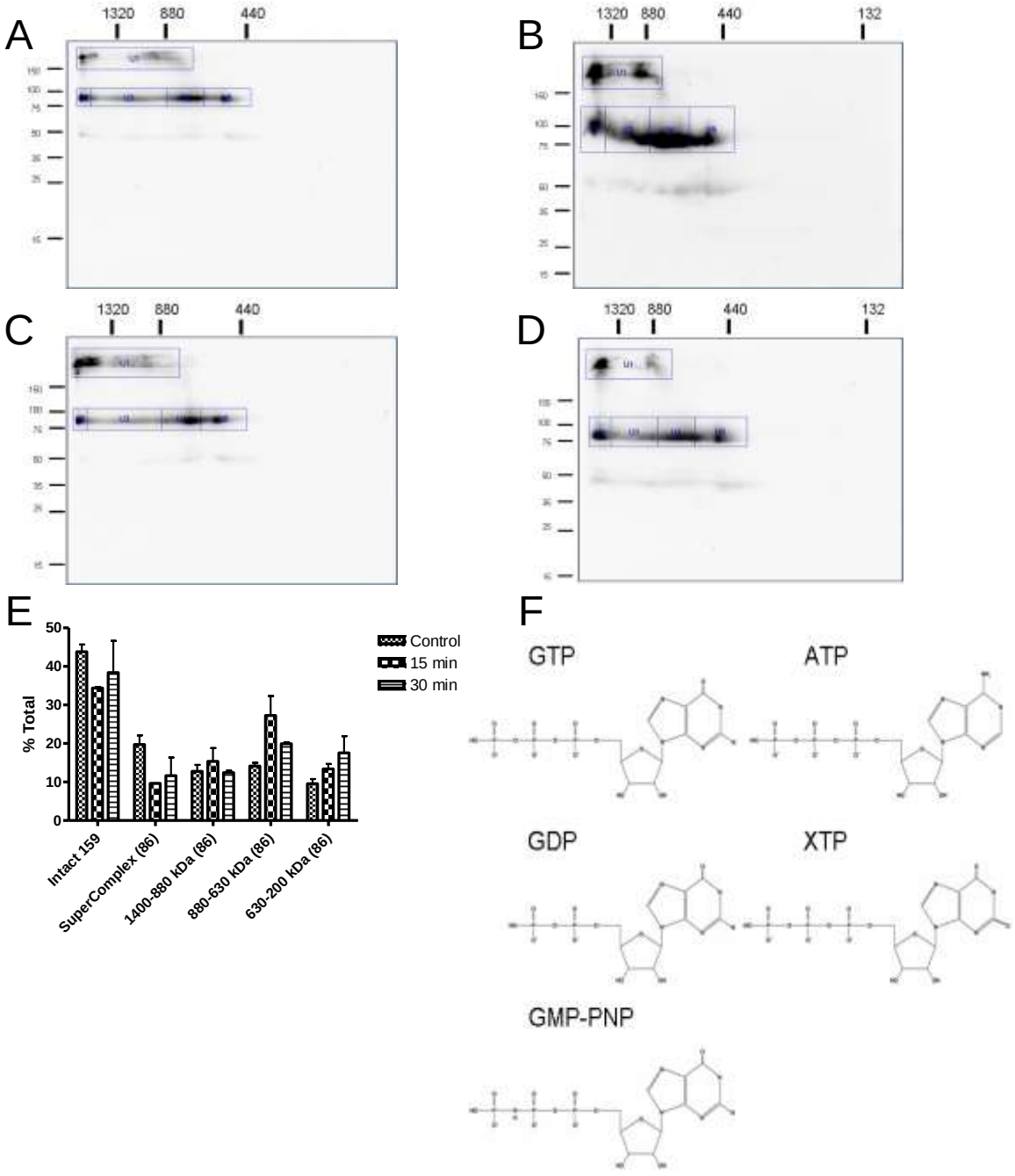
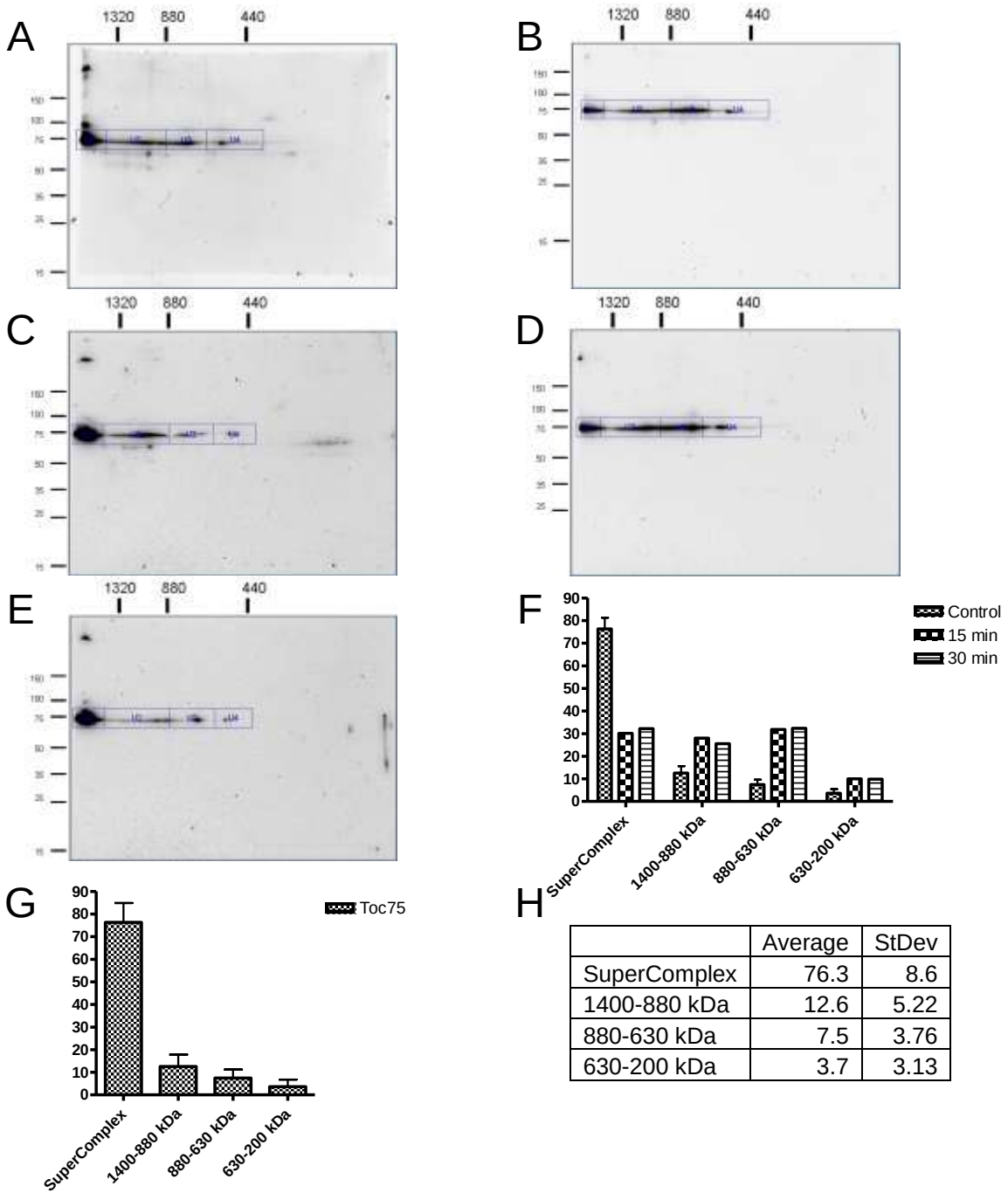


Figure 3-3 Two-dimensional BN/SDS-PAGE analysis of Toc159 distribution with GDP incubation.

A & C and B & D are from separate chloroplast preps and 1d gels. The controls without added nucleotide are in Fig. 3-1, A & B represent 15 min incubation with GDP. C & D represent 30 min GDP incubation. E is the quantitation of all blots. F shows the structures of nucleotides used in the current study.



	Average	StDev
SuperComplex	76.3	8.6
1400-880 kDa	12.6	5.22
880-630 kDa	7.5	3.76
630-200 kDa	3.7	3.13

Figure 3-4 Two-dimensional BN/SDS-PAGE analysis of Toc75 distribution with GDP incubation.

A, C, & E are three replicates of western blots from 2d gels of control 1d lanes from the first chloroplast prep. B represents 15 min incubation with GDP. D represents 30 min GDP incubation. F is the quantitation of all blots. G & H represent the mean and standard deviation for the Toc75 control experiment.

In order to determine the influence of the GDP loaded energetic state of the Toc GTPases on complex stability, intact chloroplasts were incubated with 1 mM GDP in IB with 2 mM $MgCl_2$ in the dark at 25ºC for 15 min, 30 min, or control without added nucleotide (previously mentioned control experiment was performed simultaneously with the GDP experiment). The chloroplasts were solubilized, insoluble material was removed by ultracentrifugation, and Coomassie G-250 dye was added. The samples were subjected to 2d electrophoresis, transferred to PVDF, and western blot hybridization was performed with α-Toc159, α-Toc75, and α-Toc34 antisera. Blots were quantitated with BioRad Quantity 1 Software. Toc159f exhibited decreased association with the Toc/Tic supercomplex and increased association with complexes <880 kDa (Fig. 3-3). Toc75 association with the supercomplex was also decreased with a concurrent increase in association with smaller complexes during GDP loading (Fig. 3-4). Toc34 does not show a significant change with GDP loading, which might be related to aberrant electrophoretic mobility in the second replicate (see Fig. 3-5 B, D, F), and therefore the graph of the quantitation for A, C, E is included in Fig. 3-5 H. This suggests that Toc34 shows increased participation in the 880-630 kDa complexes when loaded with GDP.

Young plants must import many more proteins into their chloroplasts during the greening stages (Dahlin and Cline 1991), and must have either more import complexes, or complexes with higher activity, and therefore young plants may have more Toc components participating in supercomplexes yielding higher efficiency of input. This hypothesis was tested by analyzing 7, 13, and 17 day old chloroplasts by 2d PAGE, and analyzing the distribution of the Toc components. Toc159 underwent more proteolytic degradation in older plants, so the quantitation data is presented with intact Toc159 compared to the size ranges of the 86 kDa fragment (Fig. 3-6 G) and the distribution of the 86 kDa fragment is presented without intact 159 to show the change in distribution without being skewed by the increased proteolysis (Fig. 3-6 H). The older plants have less Toc159f associated with the Toc/Tic supercomplex, and increased

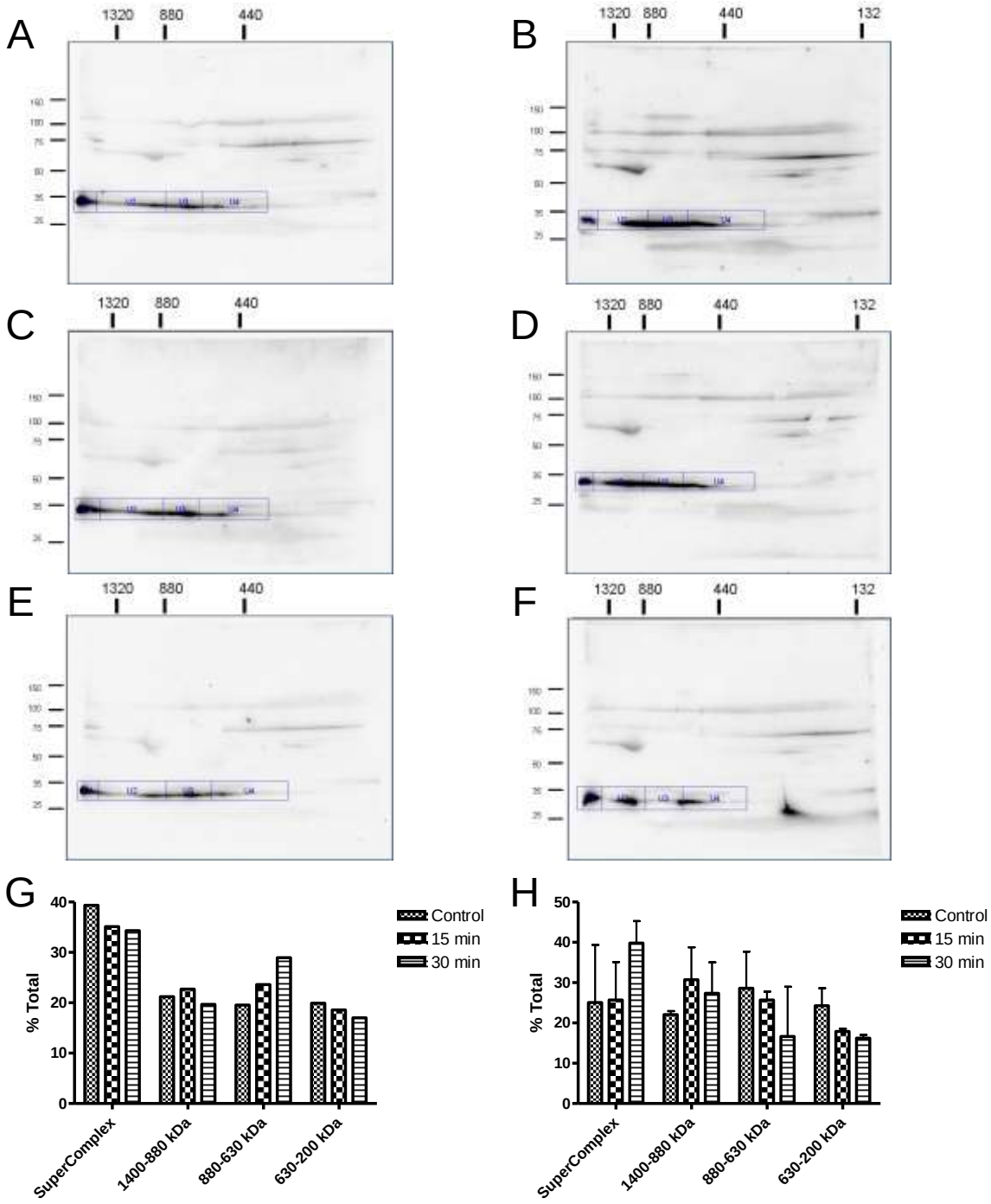


Figure 3-5 Two-dimensional BN/SDS-PAGE analysis of Toc34 distribution with GDP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with GDP. E & F represent 30 min GDP incubation. G is the quantitation of A, C, & E, and H is the quantitation of all blots.

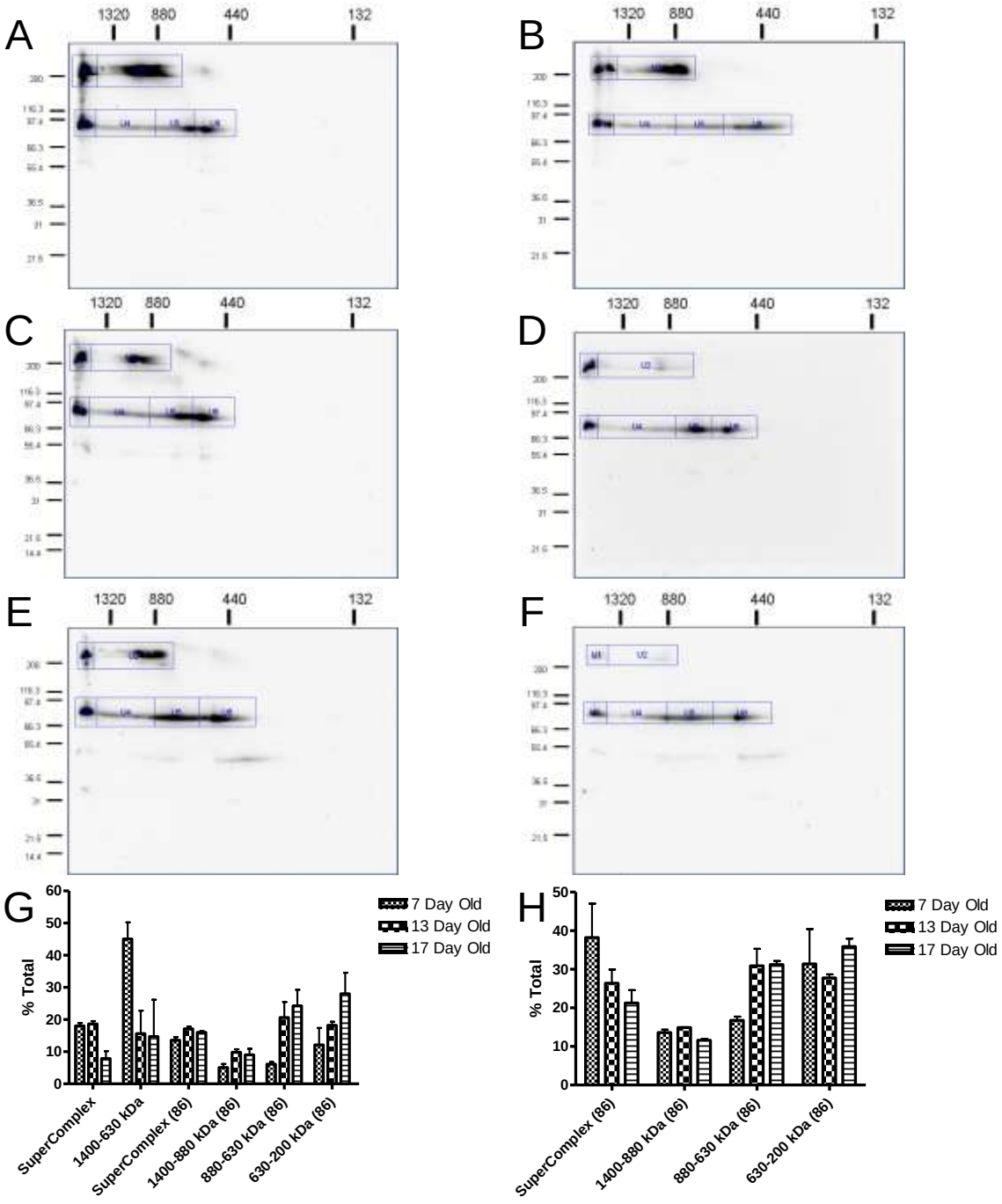


Figure 3-6 Two-dimensional BN/SDS-PAGE analysis of Toc159 distribution by age.

A, C, & E were run on one native gel and B, D, & F are from a separate 1d gel. A & B are 7 day old, C & D are 13 day old, and E & F are 17 day old. G is the quantitation of all species, and H is the quantitation of only the 86 kDa fragment.

participation in the 880-630 kDa complexes (Fig. 3-6 H). The distribution of Toc75 is unchanged with increased age (Fig. 3-7). Toc34 association with the Toc/Tic supercomplex is unchanged with age, but Toc34 association with the 1400-880 kDa complex decreases with age and participates more in the complexes less than 880 kDa (Fig. 3-8).

The GTP loaded state is the active state for most GTPases, and Toc34 shows increased preprotein binding when loaded with GTP. The influence of this energetic state on complex stability has not clearly been shown. Chloroplasts were incubated with 1 mM GTP in IB with 2 mM $MgCl_2$ in the dark at 25ºC for 15 min, 30 min, or control without added nucleotide, and analyzed by 2d PAGE. Toc159f exhibited decreased association with the 1400-880 kDa complex after 30 min GTP incubation, and the 630-200 kDa complex had a higher population of this fragment for both time points of GTP incubation in comparison to control (Fig. 3-9). The distribution of Toc75 was unchanged in response to GTP incubation (Fig. 3-10). Toc34 distribution is not significantly changed (Fig. 3-11); however, the western blot showed a large amount of noise, probably due to nonspecific binding of the antibody, and the quantitation may not be reliable.

During GTP incubation, the nucleotide may be hydrolyzed to GDP by the Toc GTPases, yielding a heterogenous mixture of GTP and GDP loaded receptors, which would make analysis of the influence of GTP on complex stability very difficult. This hydrolysis could be investigated by incubating chloroplasts with either [γ^{32}P]GTP or [α^{32}P]GTP before separation by 2d electrophoresis, transfer to PVDF, and exposing the PVDF to film. The difference in signal between the two experiments would be equivalent to the amount of GTP hydrolysis to GDP (see (Reddick, Chotewutmontri et al. 2008),(Kessler, Blobel et al. 1994)). To prevent this heterogenous mixture, the GTPases can be loaded with a GTP analog which cannot be hydrolyzed. XTP is a GTP analogue with an amine to carbonyl substitution on the purine ring. Jelic et al. (2002) have shown that Toc34 can bind, but not hydrolyze XTP. Chloroplasts were incubated with 1 mM XTP in IB with 2 mM $MgCl_2$ in the dark at 25ºC for 15 min, 30 min, or control

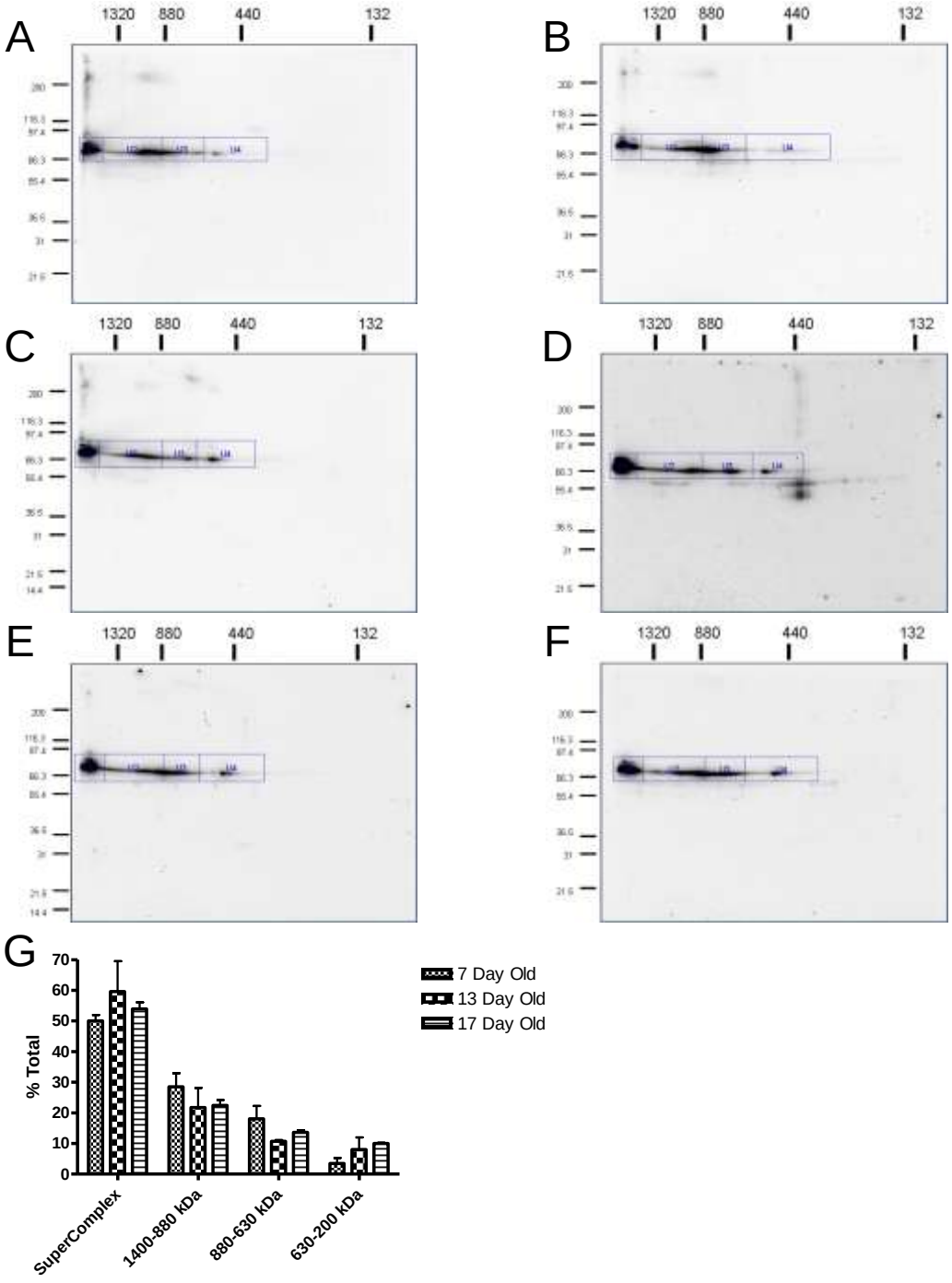


Figure 3-7 Two-dimensional BN/SDS-PAGE analysis of Toc75 distribution by age.

A, C, & E were run on one native gel and B, D, & F are from a separate 1d gel. A & B are 7 day old, C & D are 13 day old, and E & F are 17 day old. G is the quantitation of the blots.

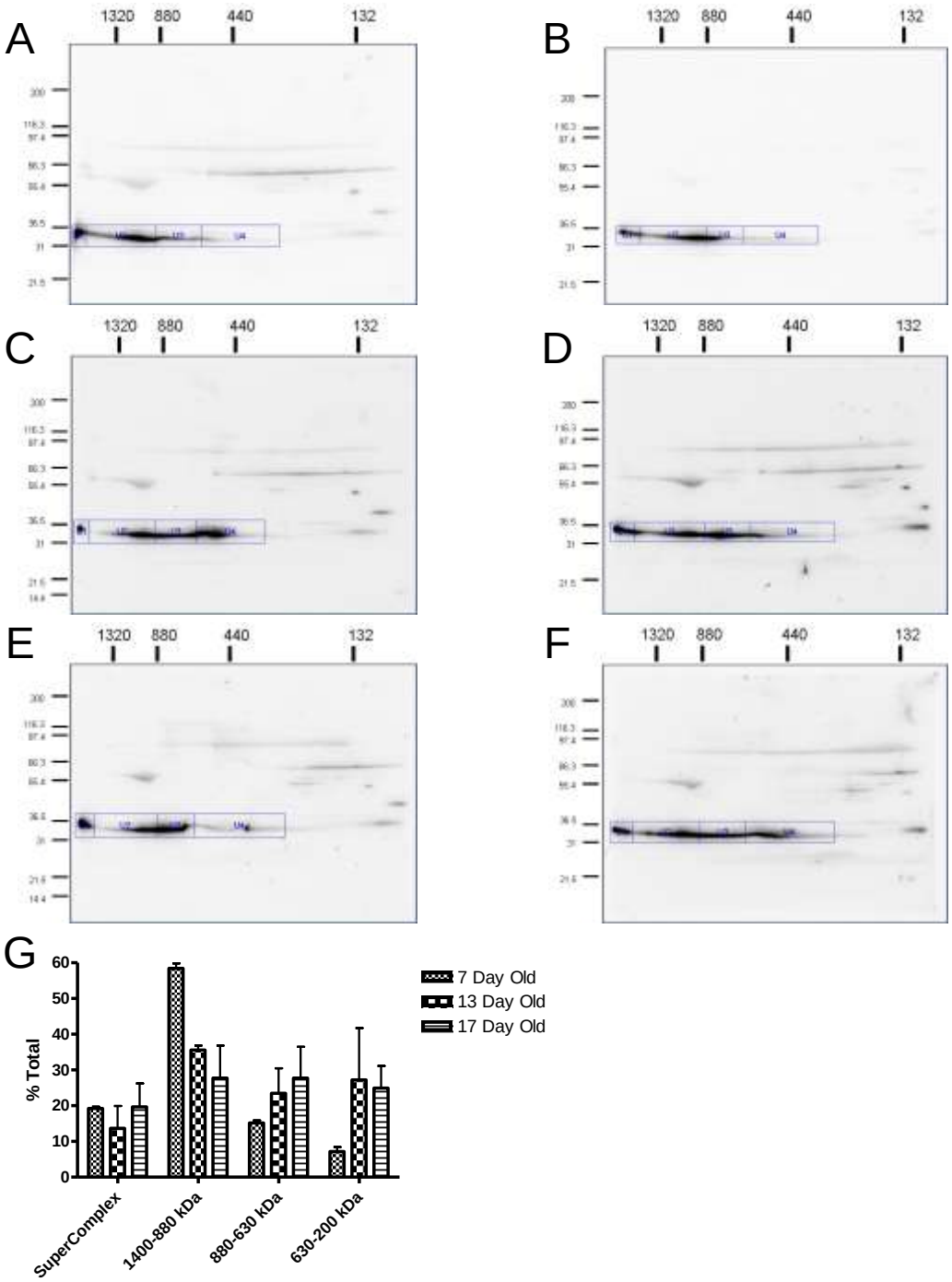


Figure 3-8 Two-dimensional BN/SDS-PAGE analysis of Toc34 distribution by age.

A, C, & E were run on one native gel and B, D, & F are from a separate 1d gel. A & B are 7 day old, C & D are 13 day old, and E & F are 17 day old. G is the quantitation of the blots.

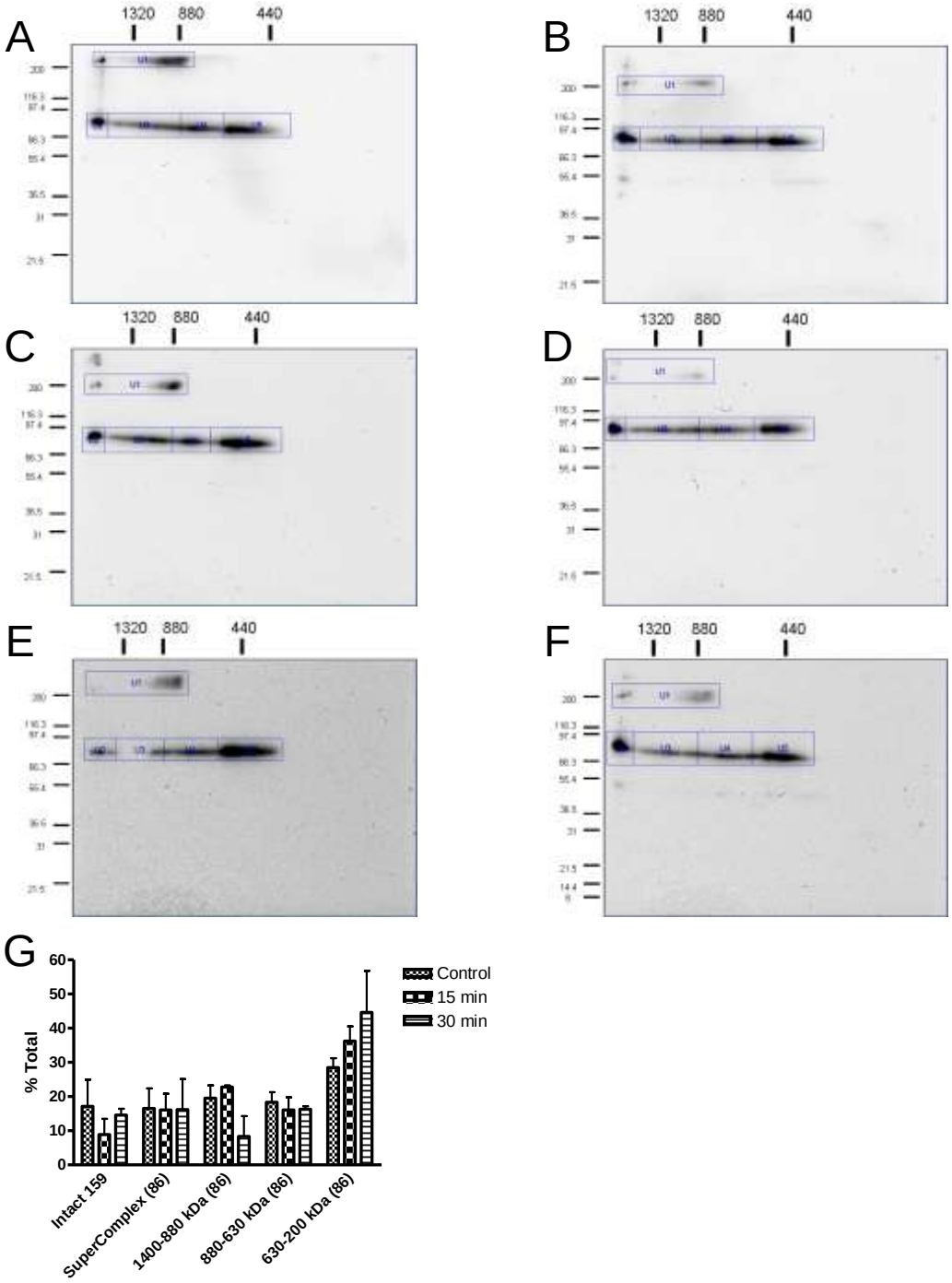


Figure 3-9 Two-dimensional BN/SDS-PAGE analysis of Toc159 distribution with GTP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with GTP. E & F represent 30 min GTP incubation. G is the quantitation of the blots.

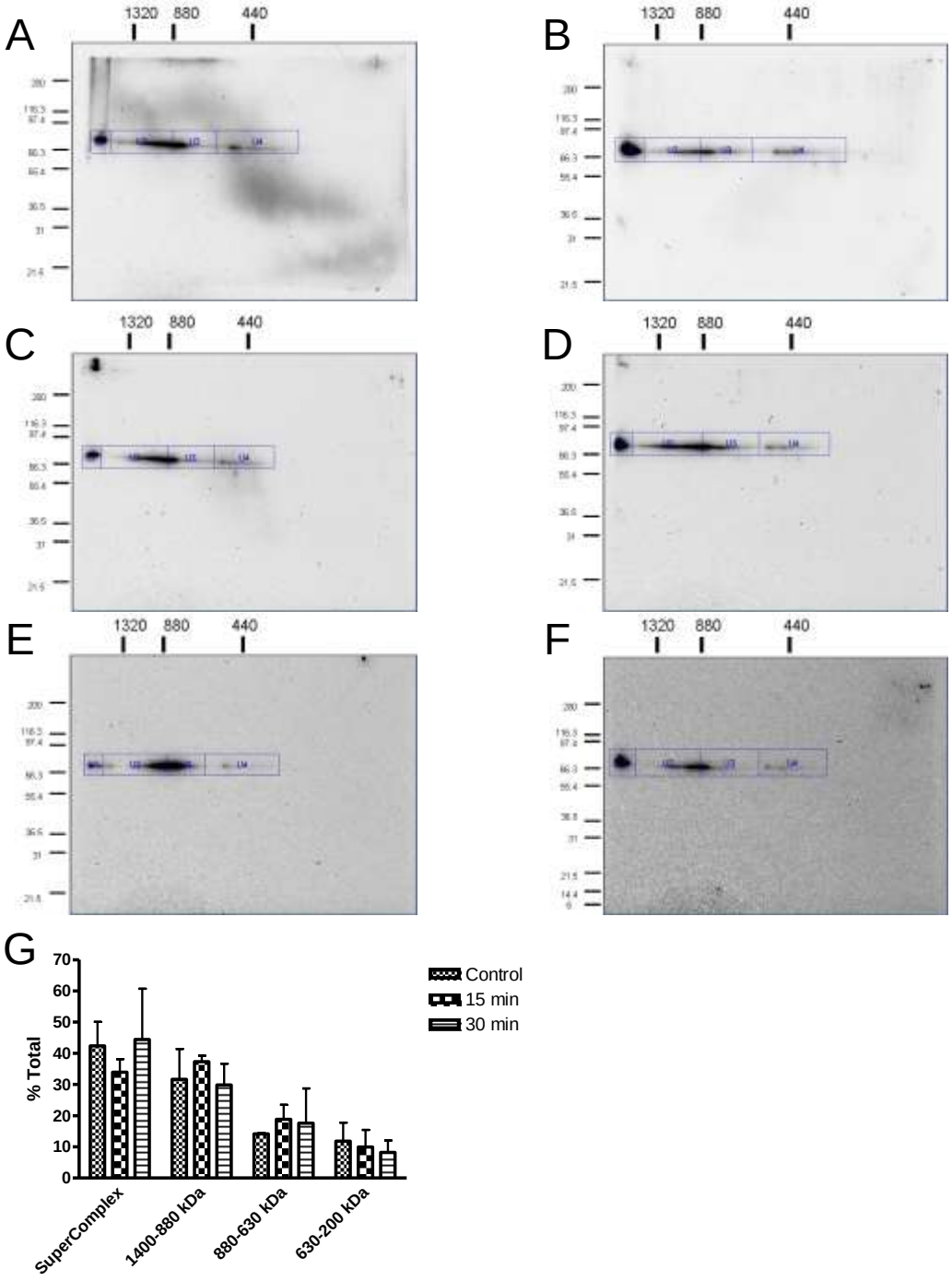


Figure 3-10 Two-dimensional BN/SDS-PAGE analysis of Toc75 distribution with GTP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with GTP. E & F represent 30 min GTP incubation. G is the quantitation of the blots.

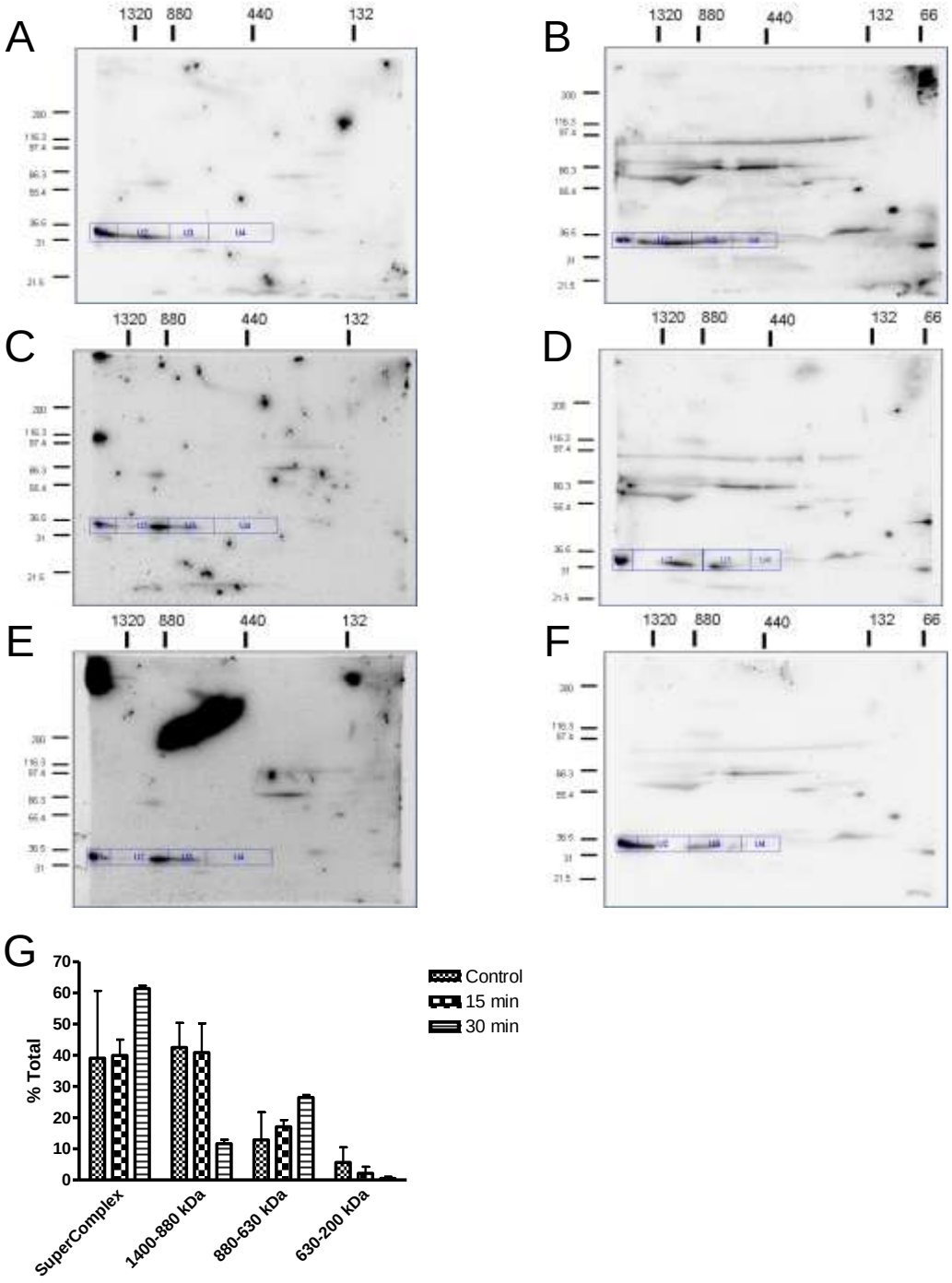


Figure 3-11 Two-dimensional BN/SDS-PAGE analysis of Toc34 distribution with GTP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with GTP. E & F represent 30 min GTP incubation. G is the quantitation of the blots.

without added nucleotide, and analyzed by 2d PAGE. There was considerable variation in the amount of intact Toc159 in the 1400-630 kDa range, possibly due to aberrant electrophoresis, which influenced the complex to total ratio, and the quantitation data was therefore also presented excluding this complex (Fig. 3-12, H). XTP incubation does not induce a significant shift in the distribution of Toc159 (Fig. 3-12). The distribution of Toc75 is not significantly changed, but association may shift slightly to smaller complexes with XTP incubation (Fig. 3-13). XTP incubation does not induce a significant shift in the distribution of Toc34 (Fig. 3-14).

GMP-PNP is a non-hydrolyzable GTP analog with a nitrogen substituted for the oxygen between the β and γ phosphate groups. Reddick et al. (2007) have shown that Toc34 binds GMP-PNP with a high affinity. Chloroplasts were incubated with 1 mM GMP-PNP in IB with 2 mM $MgCl_2$ in the dark at 25ºC for 15 min, 30 min, or control without added nucleotide, and analyzed by 2d PAGE. GMP-PNP incubation resulted in decreased association of the Toc159f with complexes in the 1400-880 kDa range and increased association with complexes below 630 kDa (Fig. 3-15). GMP-PNP incubation induces a shift in Toc75 association from participation in 1400-880 kDa complexes to association with the Toc/Tic supercomplex (Fig. 3-16). GMP-PNP does not induce a significant shift in the distribution of Toc34 (Fig. 3-17).

The hydrolysis of GTP by Toc34 and Toc159 may have implications for the oligomeric status of the Toc complex, and can be investigated with the transition state analog AlF_4. In order to determine this influence, chloroplasts were incubated with 1 mM AlF_4 in IB with 2 mM $MgCl_2$ in the dark at 25ºC for 30 min, 60 min, or control without added nucleotide, and analyzed by 2d PAGE. The analog was incubated for an extended period of time in relation to the other nucleotide treatments due to personal communication (E. Reddick) indicating the necessity of longer incubation due to performance in in vitro GTP hydrolysis assays. The incubation of chloroplasts with the transition state analog did not

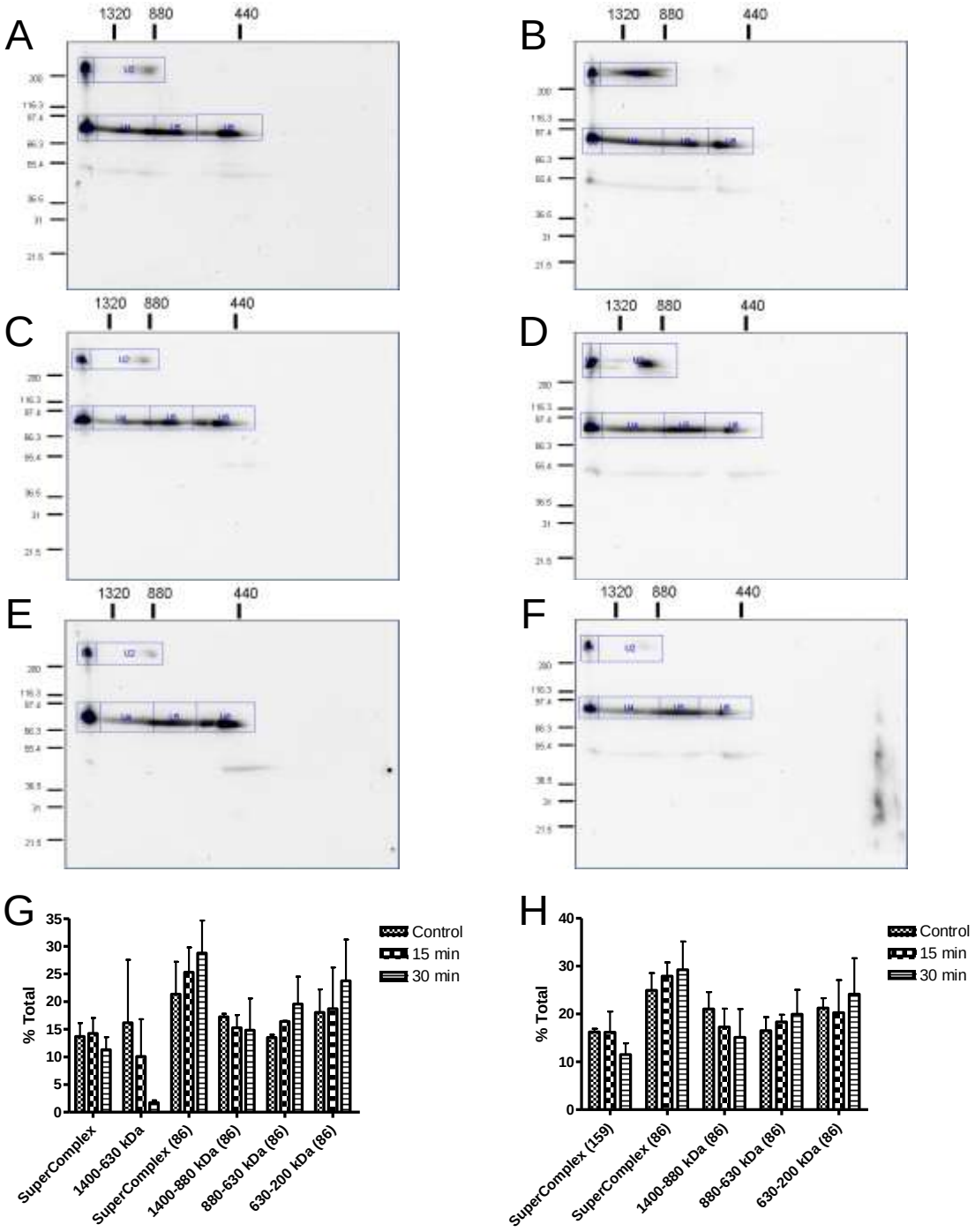


Figure 3-12 Two-dimensional BN/SDS-PAGE analysis of Toc159 distribution with XTP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with XTP. E & F represent 30 min XTP incubation. G is the quantitation of all species, and H is the quantitation excluding intact Toc159 from 1400-630 kDa.

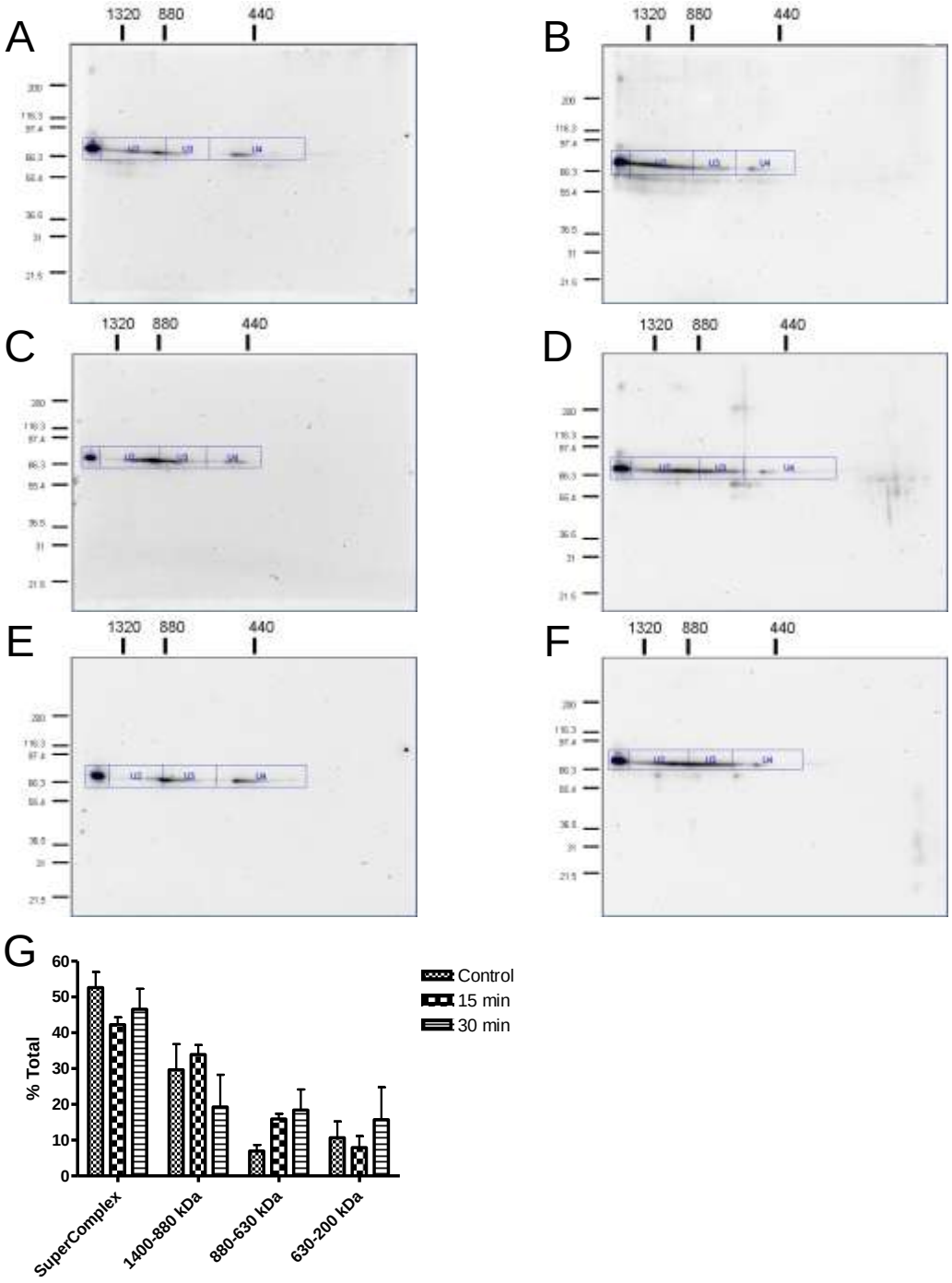


Figure 3-13 Two-dimensional BN/SDS-PAGE analysis of Toc75 distribution with XTP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with XTP. E & F represent 30 min XTP incubation. G is the quantitation of the blots.

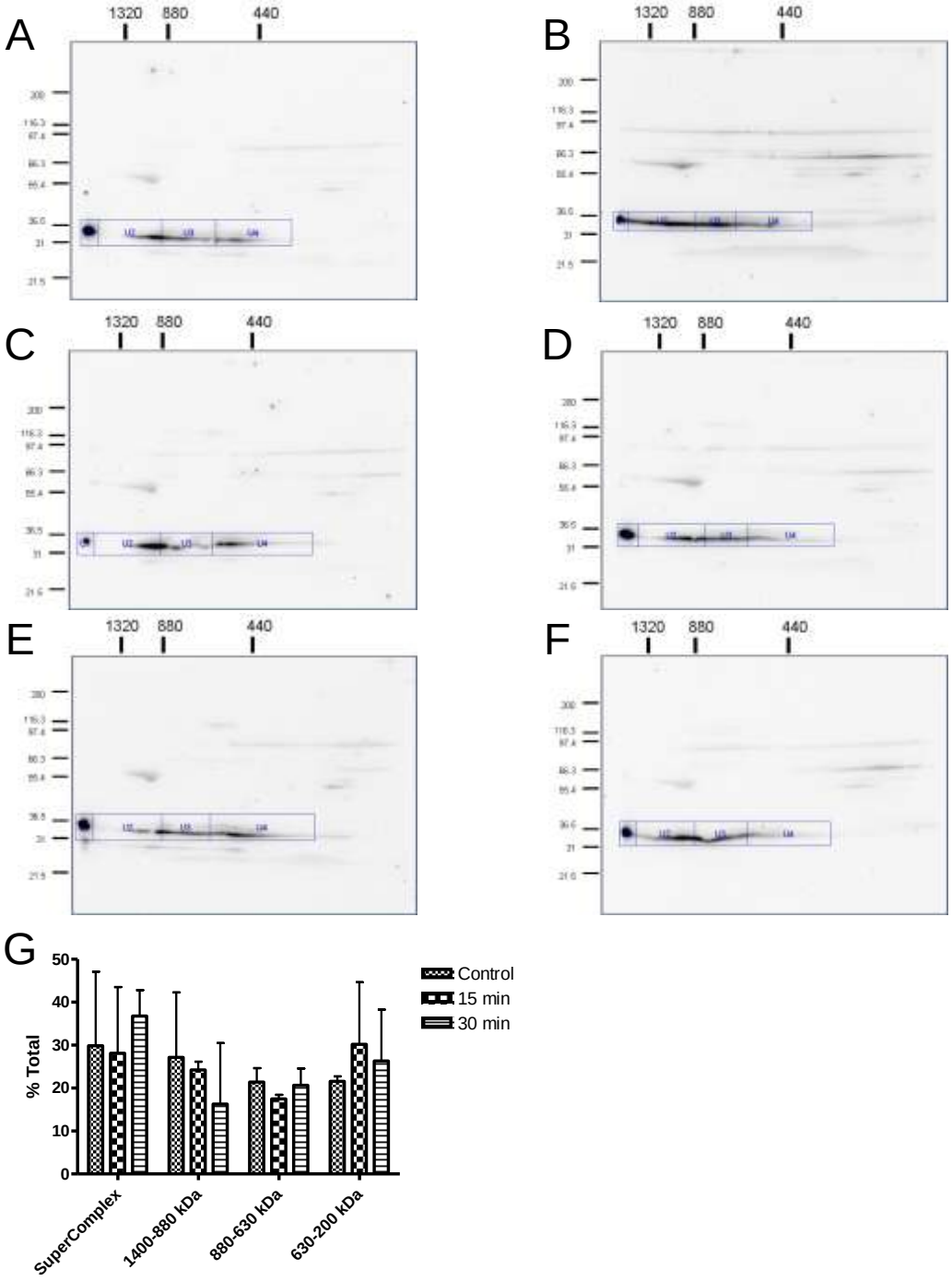


Figure 3-14 Two-dimensional BN/SDS-PAGE analysis of Toc34 distribution with XTP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with XTP. E & F represent 30 min XTP incubation. G is the quantitation of the blots.

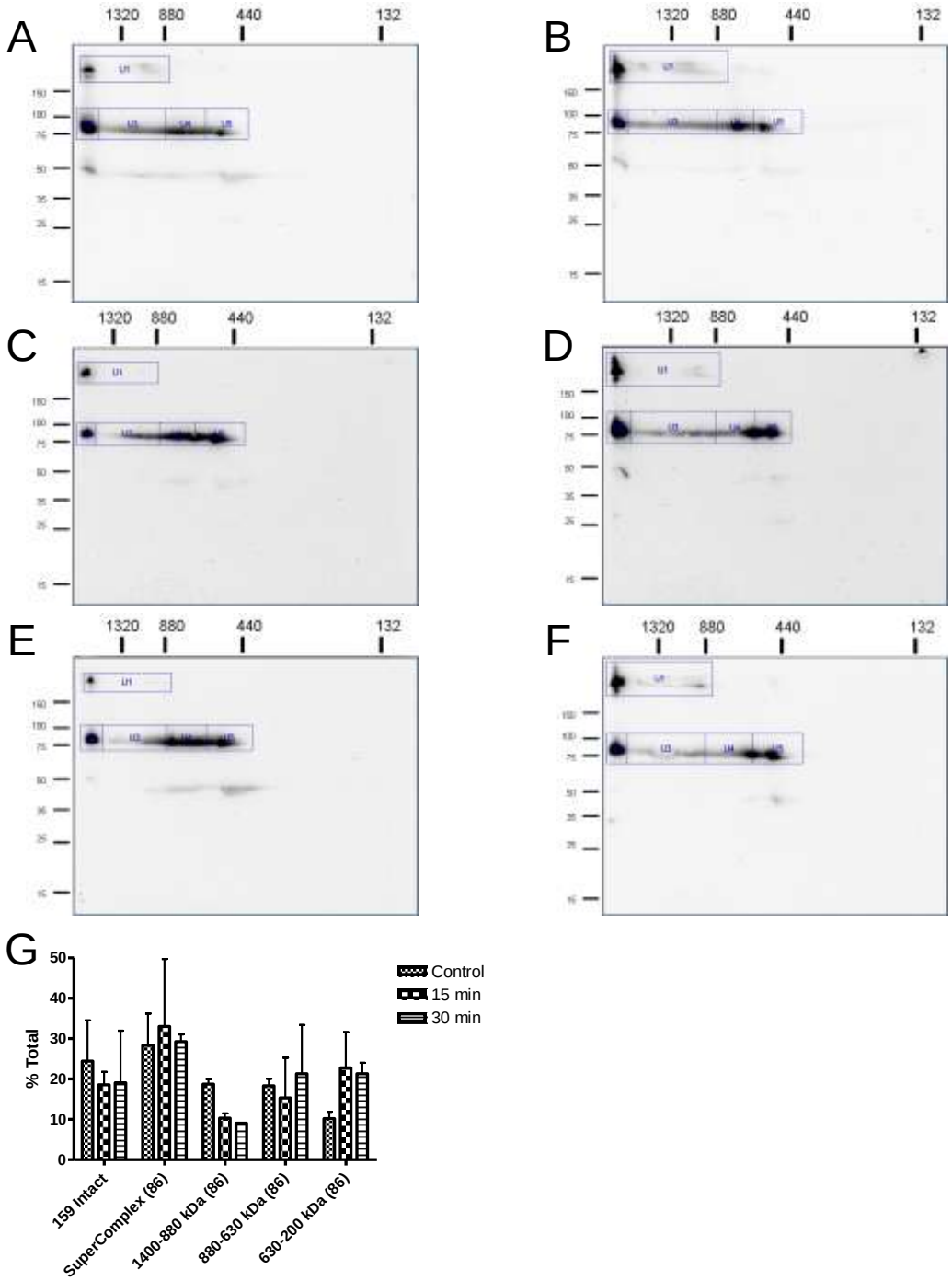


Figure 3-15 Two-dimensional BN/SDS-PAGE analysis of Toc159 distribution with GMP-PNP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with GMP-PNP. E & F represent 30 min GMP-PNP incubation. G is the quantitation of the blots.

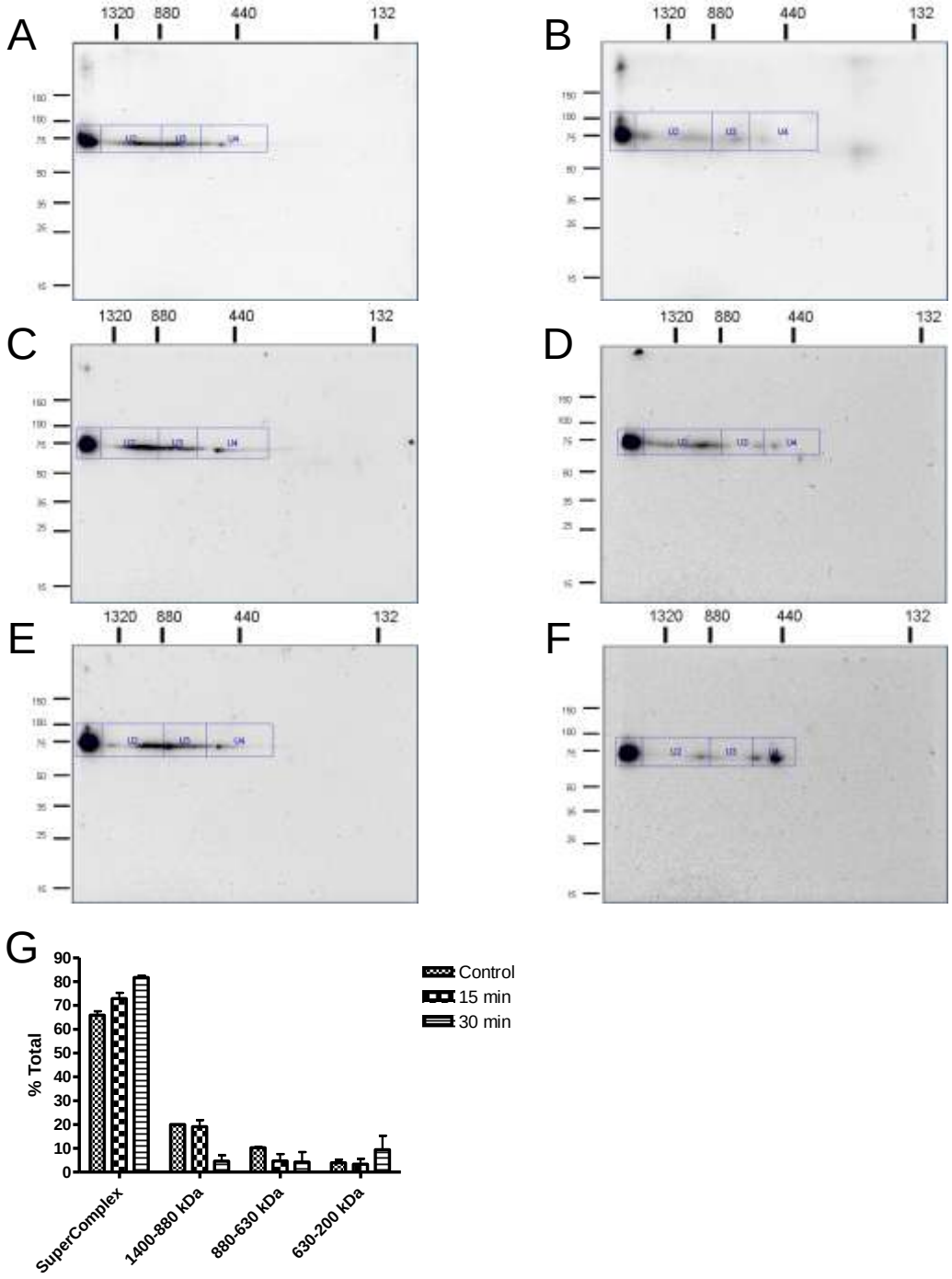


Figure 3-16 Two-dimensional BN/SDS-PAGE analysis of Toc75 distribution with GMP-PNP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with GMP-PNP. E & F represent 30 min GMP-PNP incubation. G is the quantitation of the blots.

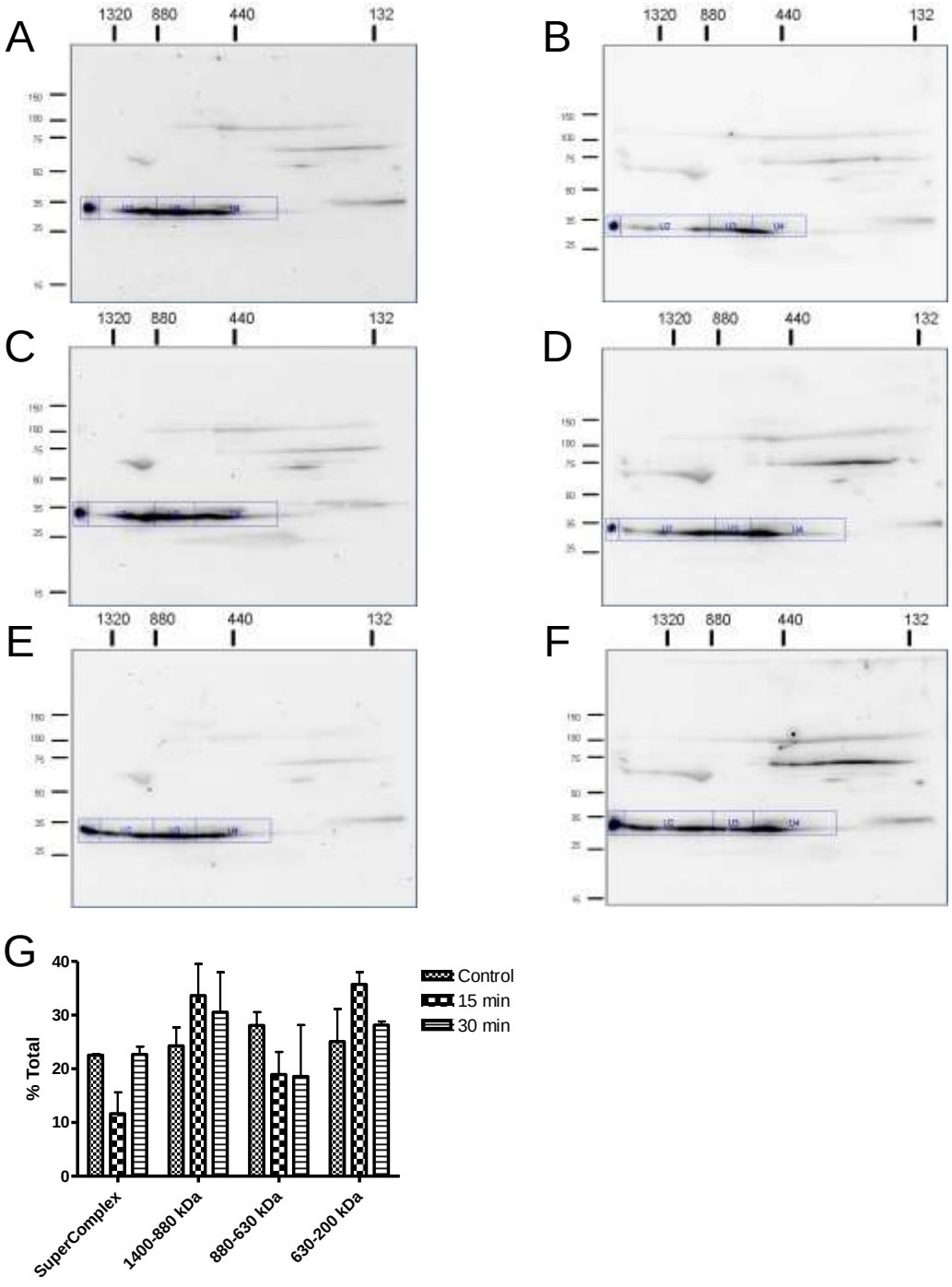


Figure 3-17 Two-dimensional BN/SDS-PAGE analysis of Toc34 distribution with GMP-PNP incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide, C & D represent 15 min incubation with GMP-PNP. E & F represent 30 min GMP-PNP incubation. G is the quantitation of the blots.

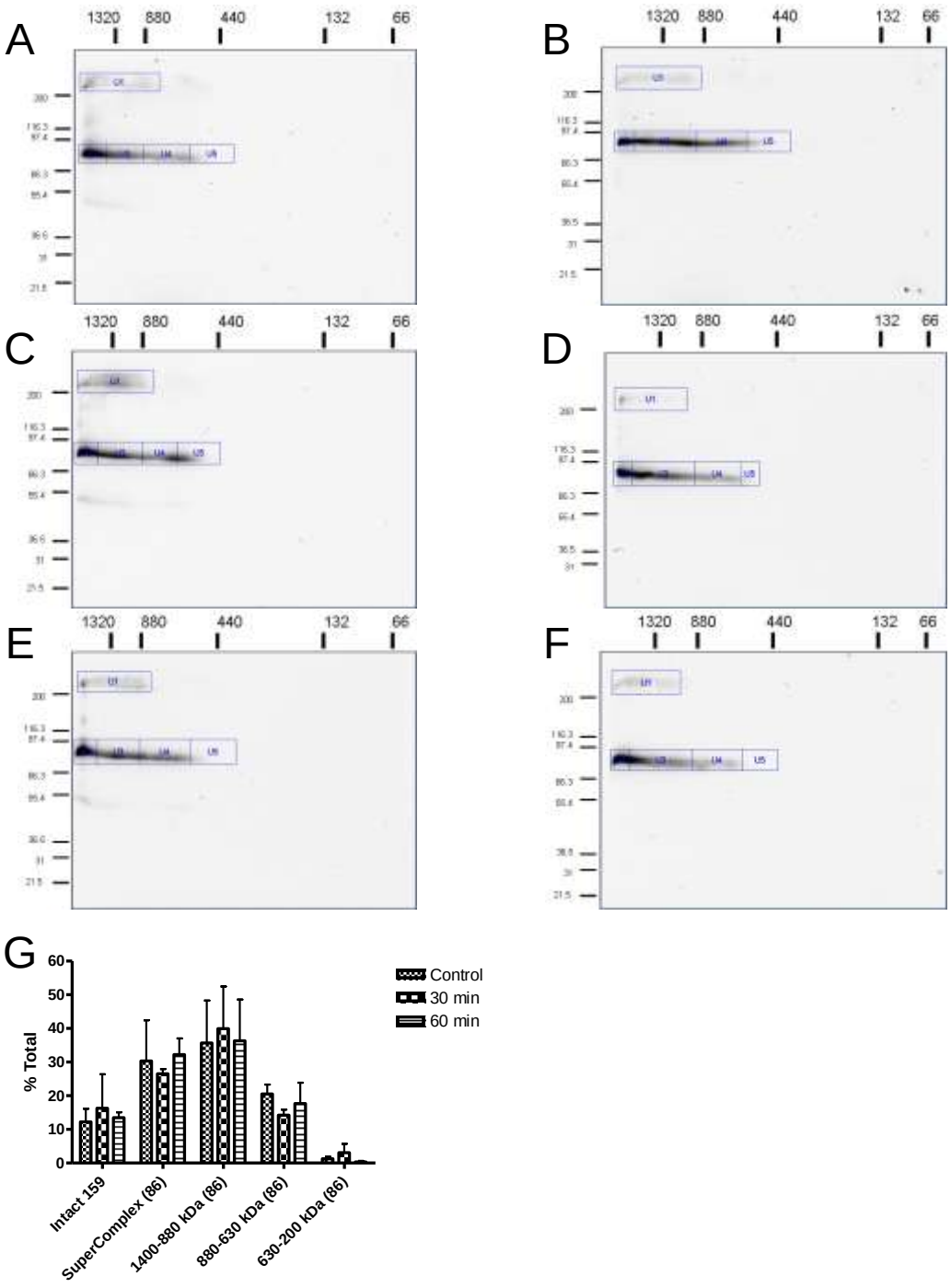


Figure 3-18 Two-dimensional BN/SDS-PAGE analysis of Toc159 distribution with AlF_4 incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added AlF_4, C & D represent 15 min incubation with AlF_4. E & F represent 30 min AlF_4 incubation. G is the quantitation of the blots.

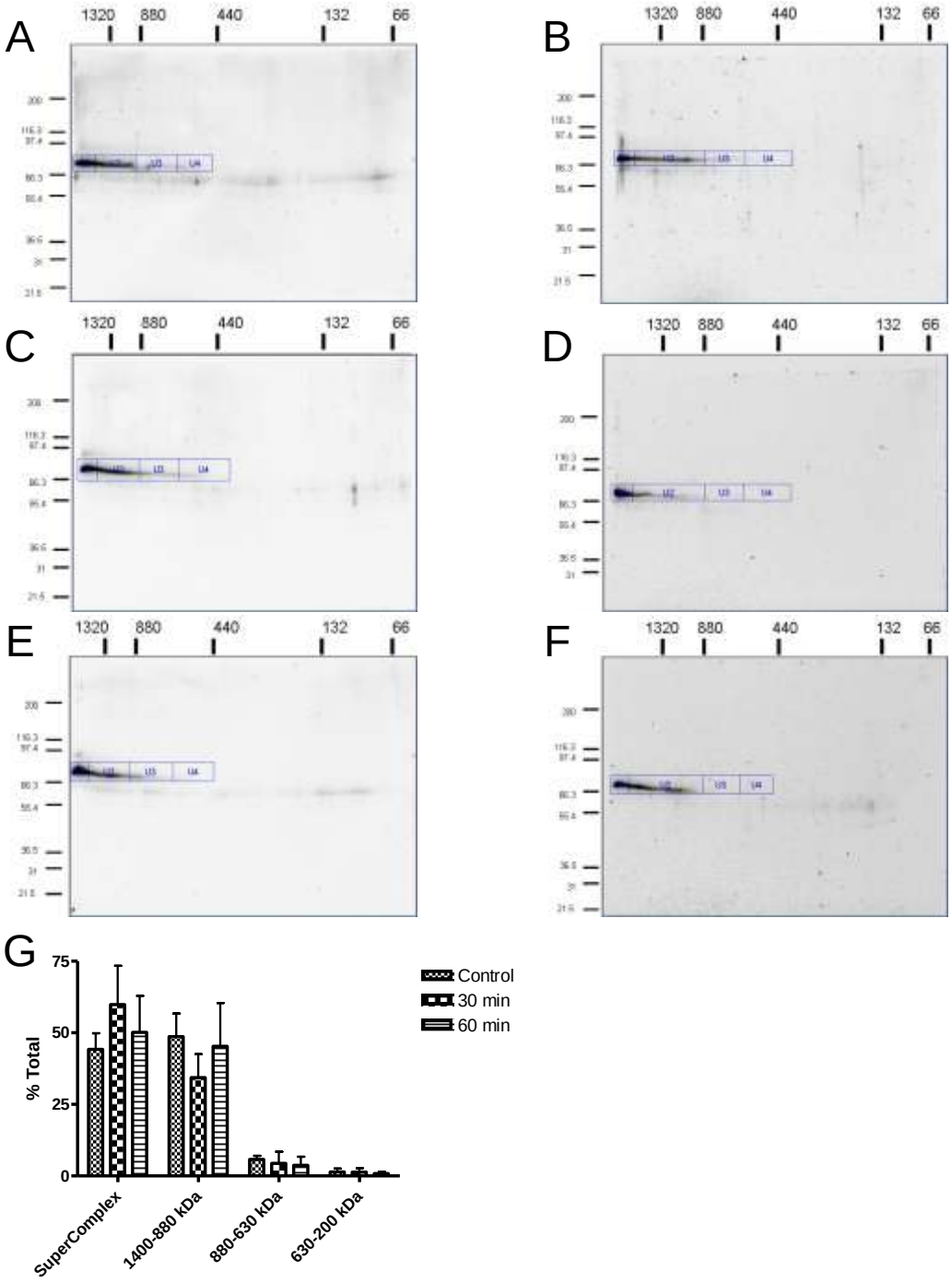


Figure 3-19 Two-dimensional BN/SDS-PAGE analysis of Toc75 distribution with AlF_4 incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added AlF_4, C & D represent 15 min incubation with AlF_4. E & F represent 30 min AlF_4 incubation. G is the quantitation of the blots.

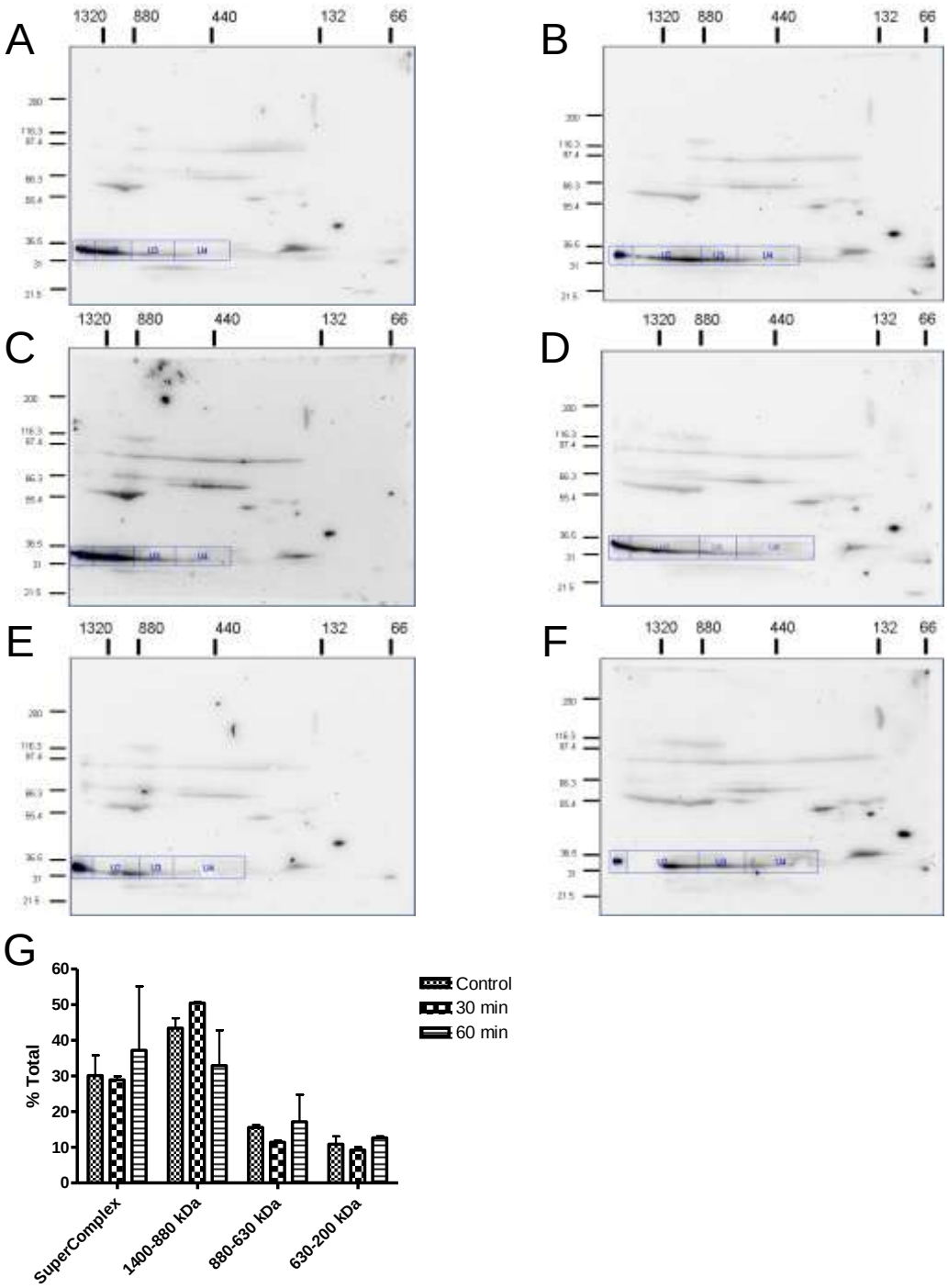


Figure 3-20 Two-dimensional BN/SDS-PAGE analysis of Toc34 distribution with AlF_4 incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added AlF_4, C & D represent 15 min incubation with AlF_4. E & F represent 30 min AlF_4 incubation. G is the quantitation of the blots.

result in a significant shift in the distribution of any of the members of the core Toc complex in comparison to controls (Fig. 3-18, 3-19, 3-20).

The import of preproteins into chloroplasts *in vitro* can be separated into three stages which are defined by differing energetic constraints. Preproteins can be reversibly bound by the receptors of the Toc complex in the absence of nucleotides in the first stage termed energy independent binding. In the presence of low levels of ATP (<0.1 mM), active binding occurs and the early import intermediate forms, in which the transit peptide of the preprotein is inserted into the channel protein Toc75, but the majority of the preprotein is still located outside of the chloroplast. This can be verified by the proteolytic degradation of the preprotein by exogenously added thermolysin, which is unable to cross the outer envelope membrane. Translocation occurs in the presence of > 1 mM ATP, and can be observed by the appearance of mature protein in SDS-PAGE due to transit peptide cleavage by the stromal processing peptidase (SPP). In order to determine the influence of active binding and import of preprotein on Toc complex oligomeric status, chloroplasts were incubated with 1.2 µM prSSU in lB in the dark at 25ºC for 15 min with 0.1 mM or 2 mM ATP with 2 mM $MgCl_2$ or control without added nucleotide or preprotein, and analyzed by 2d PAGE. Toc159f showed increased association with complexes between 880-630 kDa, but no significant shift otherwise (Fig. 3-21). Toc75 and Toc34 may exhibit a shift to increased participation in the Toc/Tic supercomplex in low and high ATP conditions, and more Toc75 is present in complexes from 880-630 kDa during binding (Fig. 3-22, 3-23).

Toc75 contains three N-terminal polypeptide transport associated (POTRA) domains, which have been suggested to function in transit peptide binding and the N-terminal region has been proposed to play a structural role in complex formation, possibly mediating the association between Toc75 and Toc34 (Sanchez-Pulido, Devos et al. 2003; Ertel, Mirus et al. 2005). In order to investigate this putative structural role, chloroplasts were incubated with excess POTRA1 (>50,000 fold excess to Toc75 in the membrane) in the dark at 25ºC for

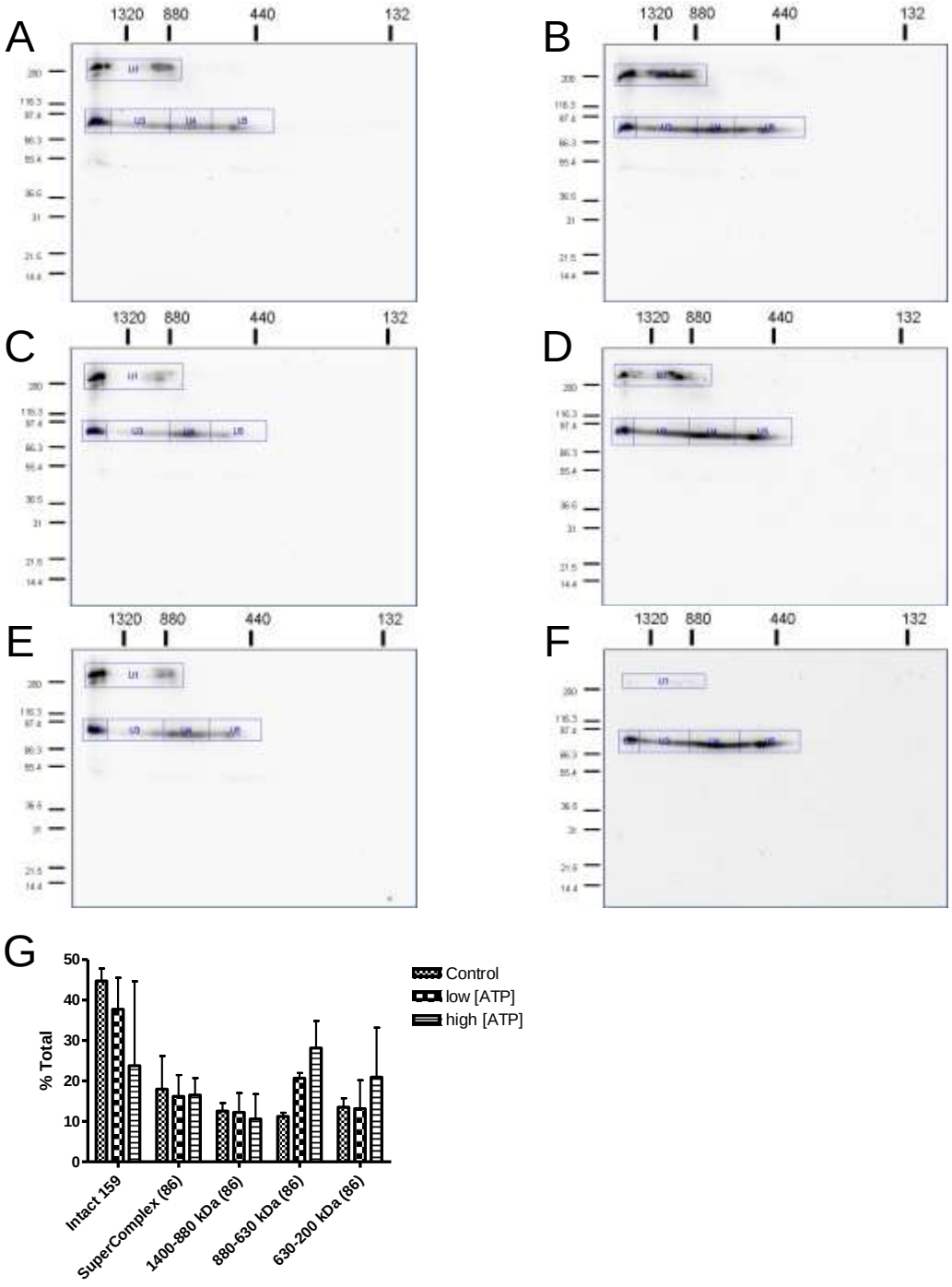


Figure 3-21 Two-dimensional BN/SDS-PAGE analysis of Toc159 distribution with prSSU incubation in binding and active import conditions.
A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide or preprotein, C & D represent 15 min incubation with prSSU with 0.1 mM ATP. E & F represent 15 min prSSU incubation with 2 mM ATP. G is the quantitation of the blots.

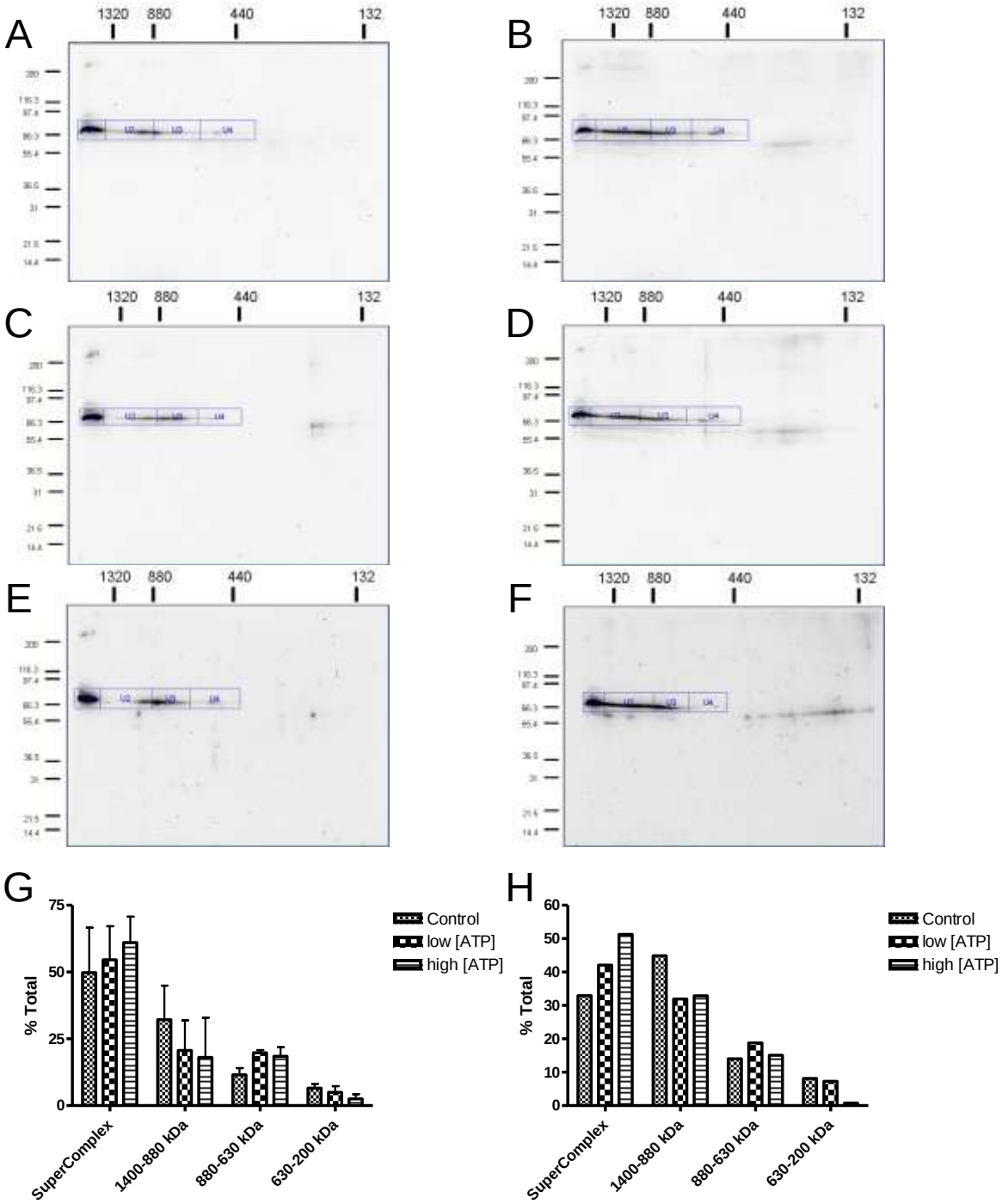


Figure 3-22 Two-dimensional BN/SDS-PAGE analysis of Toc75 distribution with prSSU incubation in binding and active import conditions.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide or preprotein, C & D represent 15 min incubation with prSSU with 0.1 mM ATP. E & F represent 15 min prSSU incubation with 2 mM ATP. G is the quantitation of all blots, and H is the quantitation of B, D, & F.

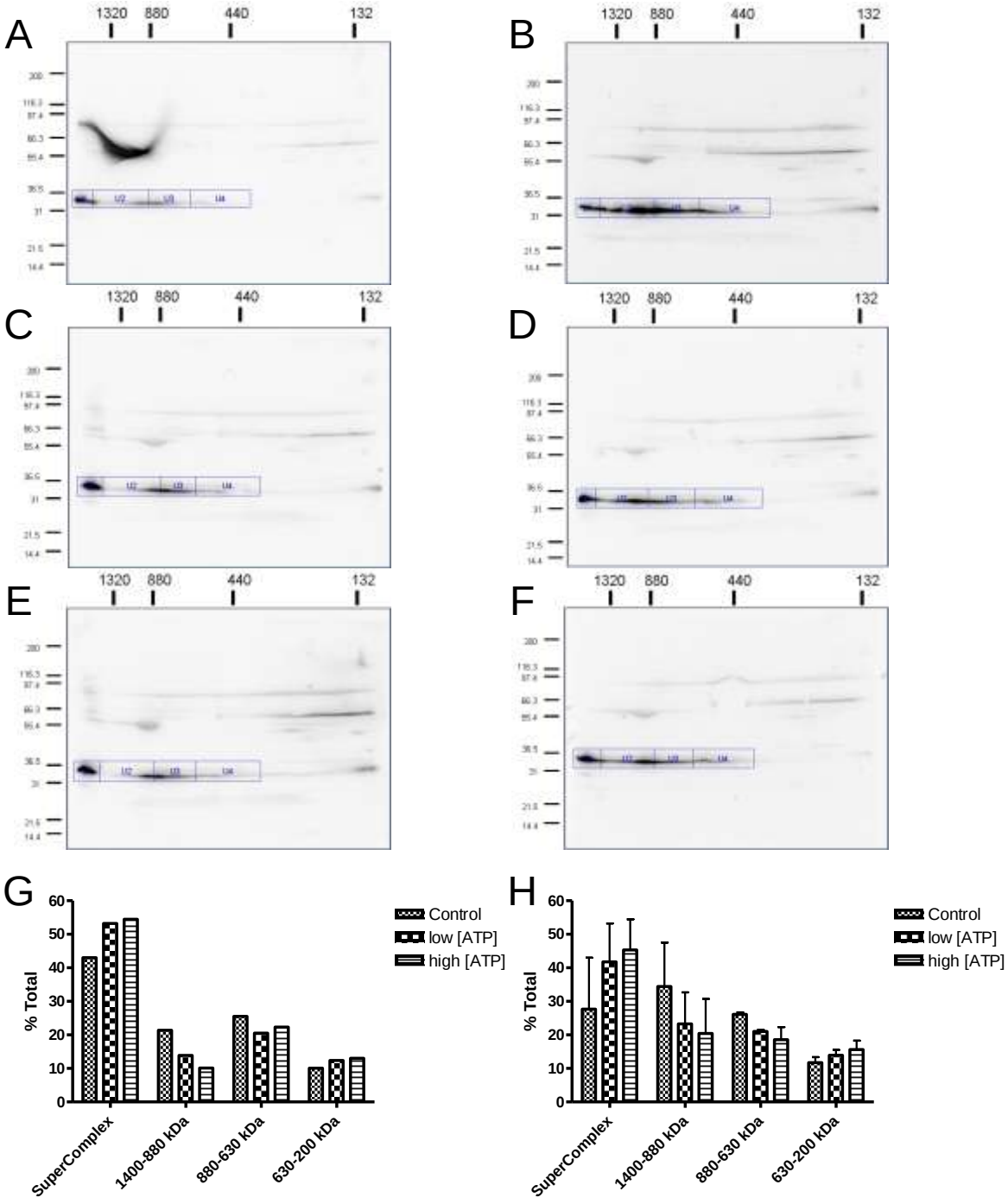


Figure 3-23 Two-dimensional BN/SDS-PAGE analysis of Toc34 distribution with prSSU incubation in binding and active import conditions.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added nucleotide or preprotein, C & D represent 15 min incubation with prSSU with 0.1 mM ATP. E & F represent 15 min prSSU incubation with 2 mM ATP. G is the quantitation of A, C, & E, and H is the quantitation of all blots.

15 min, 30 min, or control without added protein, and analyzed by 2d PAGE. A significant shift in distribution was not observed for any of the components of the core Toc complex (Fig. 3-24, 3-25, 3-26); however, the standard error of the quantitation was large due to aberrant electrophoretic migration.

3.3 Discussion

3.3.1 Chloroplasts isolated from young plants have more receptors associated with large complexes

Chloroplasts develop from small undifferentiated proplastids, and through the course of this differentiation the plastids can increase in size greater than 100-fold and proteins, lipids, and cofactors necessary for photosynthesis accumulate (Mullet 1988). The chloroplast import apparatus must therefore be able to accommodate the increase in the expression of key photosynthetic proteins. The protein import capability of chloroplasts decreases over the course of development, and has been experimentally determined to decline as much as 20-fold in *in vitro* import assays (Dahlin and Cline 1991). Chloroplasts isolated from young pea plants have more Toc159 associated with the Toc/Tic supercomplex and more intact Toc159 participating in complexes in the 1400-630 kDa range (Fig. 3-6). Young plants have more Toc34 associated with complexes in the 1400-880 kDa range (Fig. 3-8), but the distribution of Toc75 is unchanged throughout the age ranges (Fig. 3-7). This increased association of receptors with large complexes suggests that the Toc complexes may be importing preproteins with greater efficiency due to increased binding. This experiment compares the ratio of Toc components in a particular size range to the total amount observed, which only allows for the comparison of the proportions of Toc components associated with complexes of differing sizes, but not an overall change in the number of complexes with respect to age. The calculation of the number of translocons per chloroplast with respect to age would require normalization to a static component, as chlorophyll concentration,

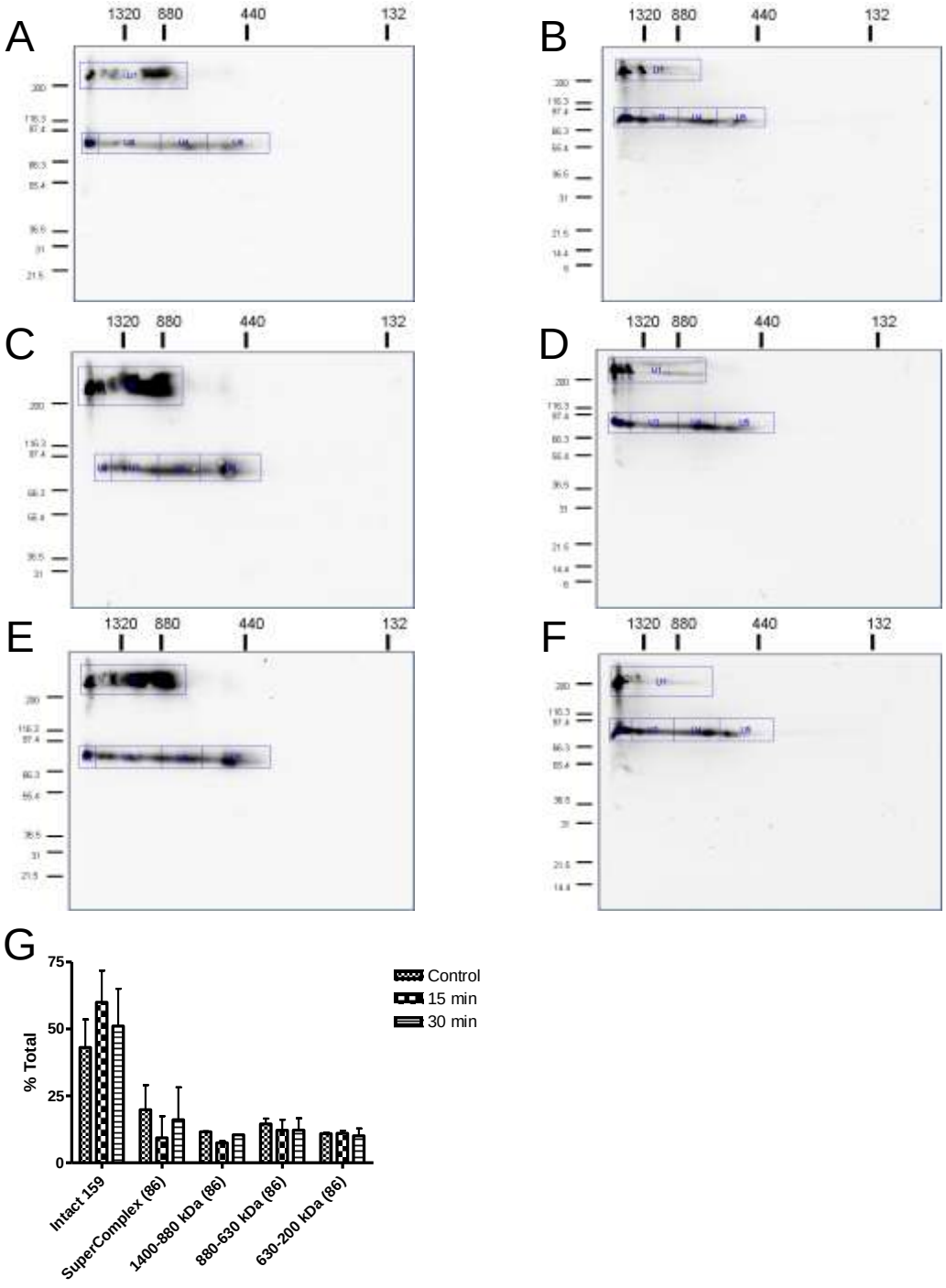


Figure 3-24 Two-dimensional BN/SDS-PAGE analysis of Toc159 distribution with POTRA1 incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added POTRA, C & D represent 15 min incubation with POTRA1. E & F represent 30 min POTRA1 incubation. G is the quantitation of the blots.

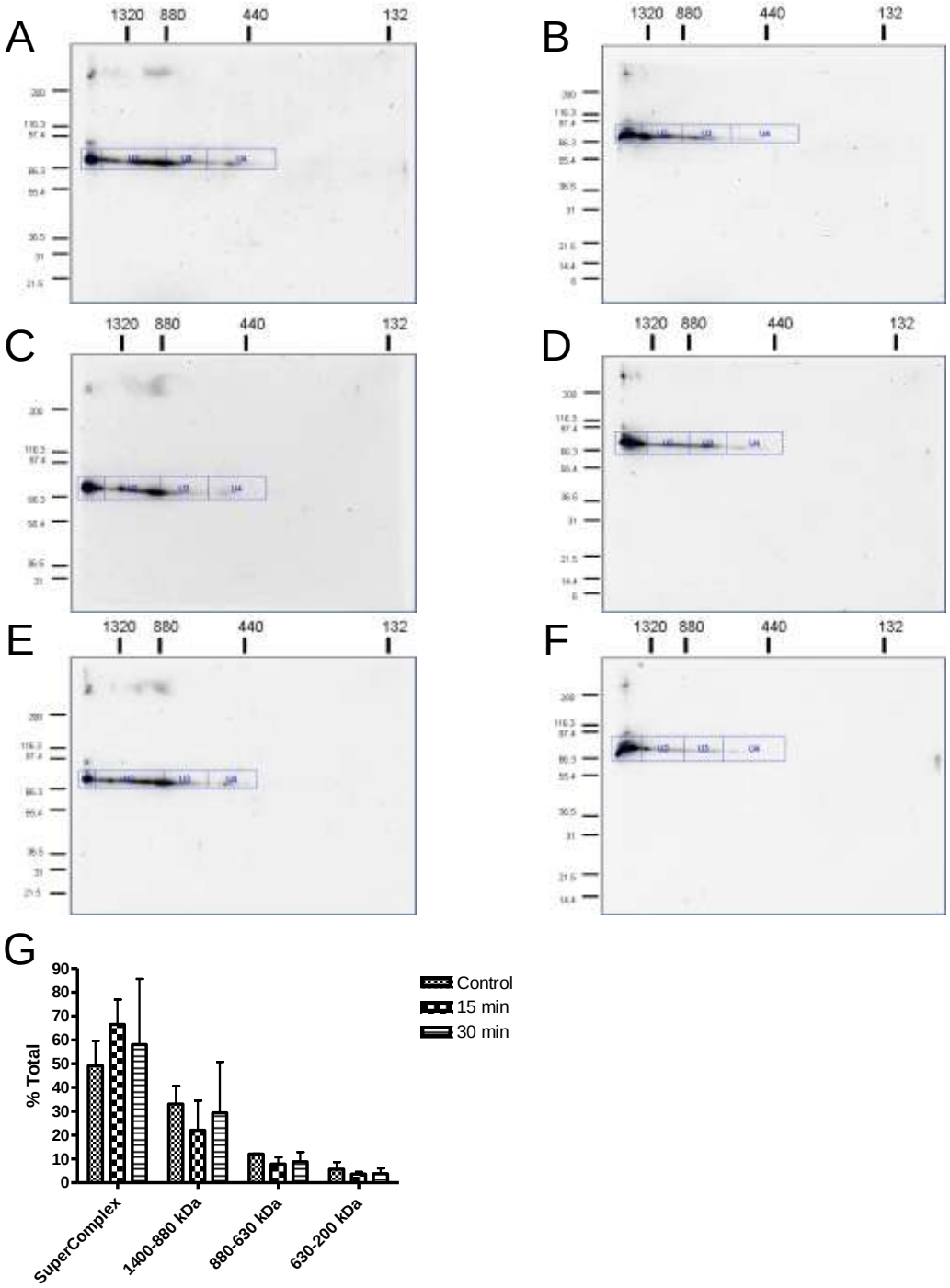


Figure 3-25 Two-dimensional BN/SDS-PAGE analysis of Toc75 distribution with POTRA1 incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added POTRA, C & D represent 15 min incubation with POTRA1. E & F represent 30 min POTRA1 incubation. G is the quantitation of the blots.

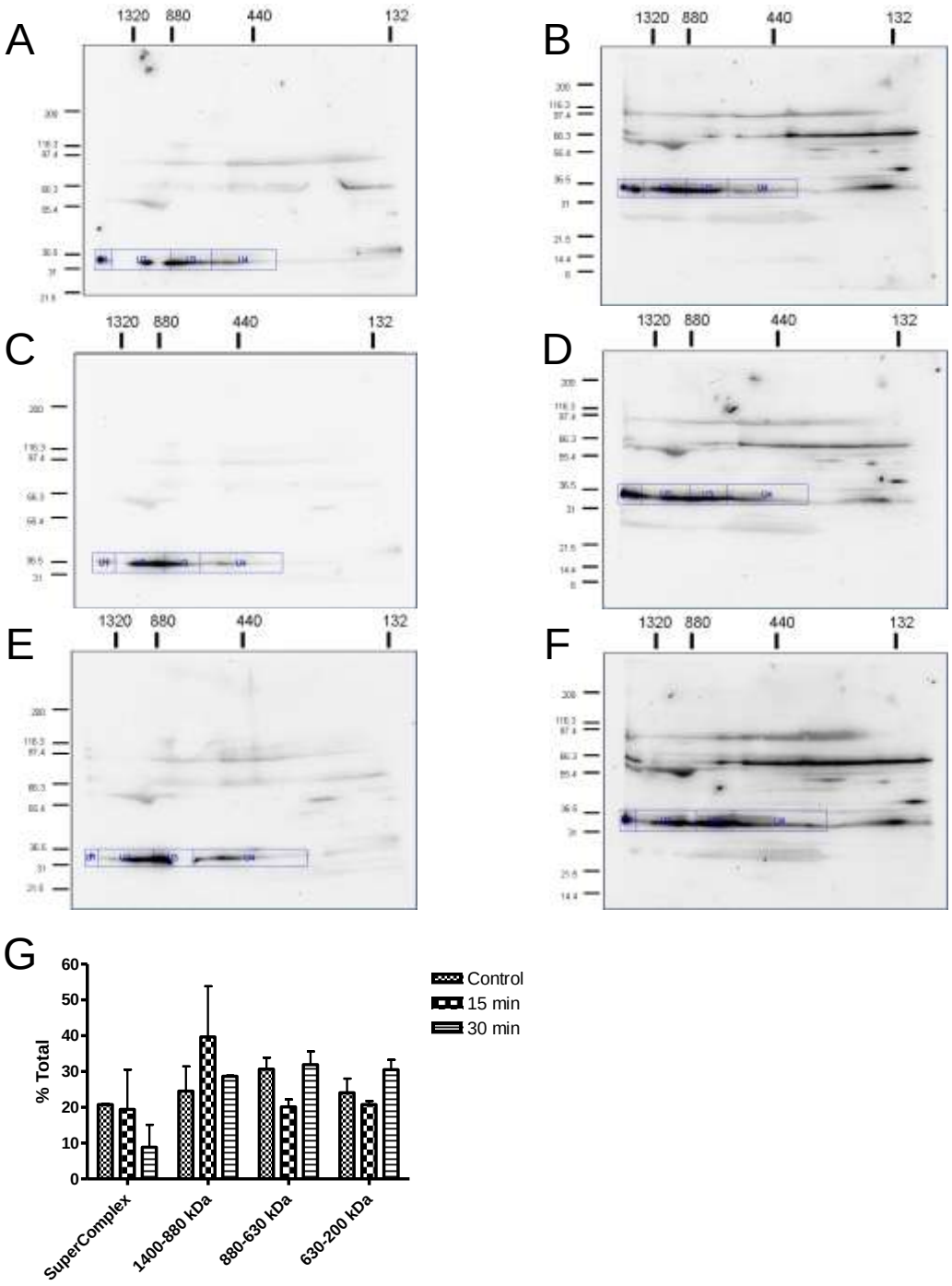


Figure 3-26 Two-dimensional BN/SDS-PAGE analysis of Toc34 distribution with POTRA1 incubation.

A, C, & E and B, D, & F are from separate chloroplast preps and 1d gels. A & B are controls without added POTRA, C & D represent 15 min incubation with POTRA1. E & F represent 30 min POTRA1 incubation. G is the quantitation of the blots.

total chloroplast protein, and other pigments such as carotenoids will change with age.

3.3.2 Nucleotide loading affects Toc complex stability

The core Toc complex is composed of the β-barrel channel protein Toc75 and the two GTPase receptors Toc34 and Toc159. The isolated soluble GTPase domains have been shown to be able to homo and heterodimerize *in vitro*, and this dimerization is stimulated by the binding of GDP. Immunoprecipitation studies have suggested that the Toc complex is destabilized by the presence of GDP in comparison to GMP-PNP or no nucleotide. These seemingly contradictory effects of GDP on the interaction of Toc components dictated further study of the nucleotide dependence of these interactions. Chloroplasts were isolated after at least 10 hours of dark conditioning in order to deplete the levels of endogenous nucleotide triphosphates. All manipulations were carried out on ice in the dark to minimize nucleotide triphosphate production by photophosphorylation. Intact chloroplasts were incubated in the dark at 25ºC with 1 mM of various nucleotides and analogs along with the cofactor Mg^{2+} for 15 or 30 minutes. The nucleotide induced instability or oligomerization was investigated by 2d PAGE.

The incubation of chloroplasts with GDP resulted in a shift in the participation of Toc75 and the 86 kDa fragment of Toc159 (Toc159f) from the Toc/Tic supercomplex to complexes <880 kDa (Fig. 3-4, 3-3), while the migration pattern of Toc34 was unchanged (Fig. 3-5). This shift suggests that Toc159 may be in a nucleotide free state before GDP incubation, as Toc159 has a higher affinity for GTP than GDP, and therefore probably would not exchange GTP for GDP unless there was a much greater excess (Reddick, Vaughn et al. 2007). If Toc159 was already in a GDP bound state, an electrophoretic shift would not be expected. The incubation of GTP does not lead to a shift in the migration of Toc75 (Fig. 3-10), but Toc159f and Toc34 show decreased participation in complexes in the 1400-880 kDa range (Fig. 3-9, 3-11). The GTP loaded state

would be the active conformation for the receptors, with high affinity for the transit peptide. A decrease in the amount of the receptors in complexes from 1400-880 suggests that GTP binding may induce a conformational change which results in the dissociation of members of the Tic complex, such as Tic110 or Hsp93. Preprotein binding would result in GTP hydrolysis, which would allow dissociation of the transit peptide from the receptors, and would allow the transit peptide to insert into the channel, engage members of the Tic complex, and proceed through translocation.

Some GTP may be hydrolyzed to GDP during the incubation period, which would mask the effects of GTP on complex stability; therefore, chloroplasts were incubated with XTP, which can bind but cannot be hydrolyzed by Toc34, and the non-hydrolyzable GTP analog GMP-PNP. Incubation of XTP may lead to decreased participation of Toc159f in complexes in the 1400-880 kDa range (Fig. 3-12), but not above the standard error. More Toc75 is present in complexes in the 880-630 kDa range with XTP incubation (Fig. 3-13), which could be explained by the dissociation of Tic components when the GTPase receptors are loaded with XTP. A shift in the mobility of the receptors would be expected as well, which can be seen for Toc159f (Fig. 3-12), but not Toc34 (Fig. 3-14) probably due to large standard error. Incubation of chloroplasts with GMP-PNP may lead to decreased participation of Toc159f in complexes in the 1400-880 kDa size range, but no changes in mobility were evident above the standard error (Fig. 3-15).

Aluminum fluoride functions as a transition state analog for hydrolysis in GTPases when present as GDP:AlF_4 in the presence of Mg^{2+}. Chloroplasts were dark conditioned for at least 10 hours prior to chloroplast isolation in order to deplete the endogenous nucleotide triphosphates. Chloroplasts were incubated with AlF_4 in the presence of 2 mM $MgCl_2$, in the assumption that the Toc GTPases would be loaded with endogenous GDP. The previous results suggest that this may not be the case for all of the receptors. Incubation of chloroplasts with AlF_4 did not lead to a significant shift in the migration pattern of any of the

Toc components (Fig. 3-18, 3-19, 3-20), which could be due to the presence of some receptors without bound GDP (either GTP bound or no nucleotide), or the lack of a conformational change during GTP hydrolysis leading to a complex stability shift or oligomerization.

The results of nucleotide induced change in Toc complex stability suggest that the formation of Toc complexes containing Tic components is not favored in the presence of GDP. GDP incubation results in the increased participation of Toc159f and Toc75 in complexes less than 880 kDa, while the 2d pattern of Toc34 is unchanged. The loading of the GTPases with GTP or non-hydrolyzable analogs results in a decrease in complexes from 1400-880 kDa. This could be caused by dissociation of Tic110 and Hsp93, Tic22, or Tic20 and dissociation of Toc GTPase homo or heterodimers. GTPases have slow hydrolysis rates without GAP, so receptors are stable in active state. GTP hydrolysis, induced by TP functioning as GAP, could therefore promote TP insertion and the recruitment of Tic components directly, or mediated through Toc75 and putative IMS domains of Tic110.

3.3.3 Size shift in Toc complex associated with preprotein import

Preproteins can reversibly bind to the chloroplast receptors in the absence of nucleotides in a process called energy-independent binding. In the presence of low levels of ATP and GTP, active binding occurs forming the early import intermediate, in which the transit peptide is deeply inserted into the Toc complex and makes contact with members of the Tic complex. GTP hydrolysis promotes the release of the preprotein from the GTPase receptors allowing insertion into the Toc75 channel. A specific role for Toc34 in protein import was initially missing, as it was not cross-linked to preproteins in early experiments (Perry and Keegstra 1994; Ma, Kouranov et al. 1996). Kouranov and Schnell (1997) showed that Toc34 cross-linking is very sensitive to the presence of nucleotide triphosphates: incubation of chloroplasts with apyrase, which catalyzes the hydrolysis of ATP to AMP and inorganic phosphate, for 10 minutes reduced

external ATP enough to allow Toc34 cross-linking, whereas a 5 minute incubation was insufficient (Ma, Kouranov et al. 1996). These results show that there is a sufficient level of endogenous GTP to allow insertion of the transit peptide into the Toc75 channel. GTP is not required during the translocation step of import. In the presence of high levels of ATP, the preprotein is translocated into the stroma where it is processed into the mature form of the protein. During active binding, the preprotein has been shown to interact with Toc75, Toc159, Toc34, Tic110, and Hsp93 by cross-linking and immunoprecipitation (Akita, Nielsen et al. 1997; Nielsen, Akita et al. 1997). During active import, preproteins interact with a very high molecular weight Toc/Tic supercomplex (Akita, Nielsen et al. 1997; Chen, Chen et al. 2000).

The influence of added preprotein on Toc complex oligomerization was examined in active binding and active import conditions by 2d PAGE. The amount of Toc75 present in the supercomplex was increased during binding and active import in comparison to control conditions (Fig. 3-22, G & H). An increase in the amount of Toc75 in the supercomplex in active import conditions is expected, as import usually proceeds across both membranes at contact sites in a concerted mechanism involving a Toc/Tic supercomplex. Toc75 and Toc159f show increased participation in complexes in the 880-630 kDa range during active binding and active import (Fig. 3-22, 3-21). Chen and Li (2007) characterized Toc complexes interacting with preprotein during active import, and determined that preproteins interact with a ~880 kDa complex containing the three Toc components, before interacting with a complex around 1320 kDa which also includes Tic110, Hsp93, and an hsp70 homologue, but not Tic40. This 1320 complex is proposed to be an intermediate between the complex containing only Toc components and the supercomplex, and that Tic40 is one of the last proteins recruited (Chen and Li 2007). Kikuchi et al. (2006) observed a complex in the size range of 350-500 kDa when Toc159 was degraded to the 86 kDa fragment. The complex studied by cryo EM by Schleiff et al. (2003) was ~500 kDa, and was composed of Toc159f, Toc75, and Toc34 in the proposed ratio 1:4:4, which

would be 522 kDa. This suggests that the complex in the 880-630 kDa range contains proteins other than the three core Toc components, or the presence of the components in a different ratio than that previously observed. The active binding of prSSU could recruit Tic110 and possibly Hsp93 or an hsp70 homologue.

3.3.4 Structural role of POTRA domains is not supported

Toc75 is a member of the Omp85 family of proteins, which are characterized by a C-terminal integral membrane β-barrel domain and an N-terminal domain rich in polypeptide-transport-associated (POTRA) repeats (Gentle, Burri et al. 2005). Toc75 contains three POTRA domains, which are predicted to contain three β-strands with the latter two separated by two α-helices (Sanchez-Pulido, Devos et al. 2003). The precise function of the POTRA domains has not been determined, but a chaperone function has been proposed (Sanchez-Pulido, Devos et al. 2003). This would explain how the Toc complex can function without the presence of chaperones (Schleiff, Jelic et al. 2003). A structural role has also been proposed, with the N-terminal POTRA containing region of Toc75 mediating the contact between Toc75 and Toc34 (Ertel, Mirus et al. 2005). In order to examine the putative structural function of the POTRA domains, intact chloroplasts were incubated with isolated POTRA1 from Toc75. If this domain has a structural function mediating contacts between members of the Toc complex, the addition of isolated POTRA domains should result in a destabilization of the Toc complex, and therefore a shift in the electrophoretic migration of the members of the Toc complex to smaller species. On the other hand, if these domains function as chaperones, binding transit peptides, then a shift in the migration of the Toc complex would not be expected. Chloroplasts incubated with an excess of POTRA1 for 15 or 30 minutes did not undergo a shift in the migration pattern of any members of the Toc complex in comparison to control without added POTRA (Fig. 3-24, 3-25, 3-26). This experiment does not support the structural function of the POTRA domains of Toc75, and suggests

they function as the low affinity binding site observed for Toc75 (Ma, Kouranov et al. 1996; Hinnah, Hill et al. 1997; Hinnah, Wagner et al. 2002).

3.4 Conclusions and Future Directions

3.4.1 Model

The results of the investigation of the effects of plant age, nucleotide loading, and the incubation of prSSU in active import conditions (2 mM ATP) are shown in the following model (Figure 3-27). As plants get older, less receptors are associated with large complexes, which results in more Toc34 participating in complexes in the 630-200 kDa range. Loading the Toc complex with GDP results in a decrease in the amount of Toc75 participating in the Toc/Tic supercomplex, which results in increased participation in complexes from 880-630 kDa which may be composed of the core Toc complex possibly associated with some Tic components. Incubation with GTP results in a shift of the participation of Toc159f from complexes in the range of 1400-880 kDa, which may be composed of the Toc core complex and Tic components or a superdimer of Toc components, to smaller complexes in the range of 630-200 kDa, which are most likely composed of the Toc core components. The incubation of chloroplasts with prSSU in active import conditions (2 mM ATP) results in a shift of participation of Toc75 from complexes in the range of 1400-880 kDa to the supercomplex where the preprotein is able to cross the outer and inner membranes simultaneously.

3.4.2 Utility of 2d electrophoresis as complex stability/oligomerization assay

Two dimensional electrophoresis, utilizing BN-PAGE in the first dimension and SDS-PAGE in the second, has been shown to be a useful system for the determination of the oligomeric status of membrane protein complexes

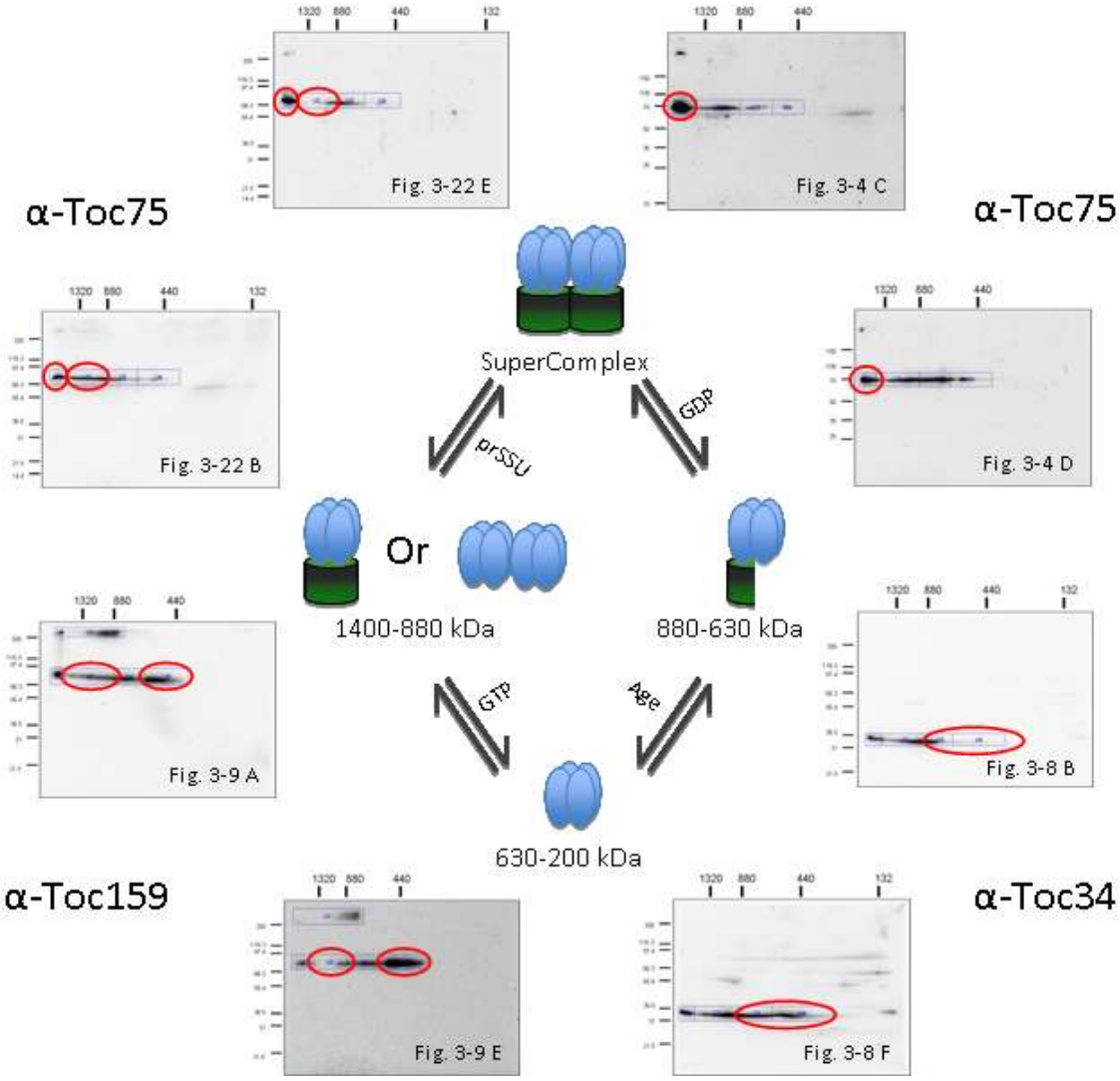


Figure 3-27 Model of Toc complex oligomerization in response to experimental conditions.

This is the summary of the effects of the experiments on Toc complex size. Experimental conditions are clockwise from upper left (Fig. 3-22 B): control and incubation with prSSU and 2 mM ATP (α-Toc75), control and incubation with 1 mM GDP (α-Toc75), chloroplasts harvested from 7 day old and 17 day old pea plants (α-Toc34), and incubation with 1 mM GTP and control (α-Toc159).

(Schagger, Cramer et al. 1994). The present study has attempted to utilize this system in combination with nucleotide and protein/peptide incubations as an assay investigating the stability and/or induced oligomerization of the Toc complex. Several problems in the protocol led to difficulties in data interpretation. There was a significant amount of variation in the 2d electrophoretic pattern of control blots from different chloroplast preps. This type of experimental setup is biochemically very complex, and variability is inherent in any complex experiment. This variation showed the absolute necessity of utilizing controls from the same chloroplast prep and 1d gel as the experimental conditions. Data from multiple preps can be combined, and overall trends are apparent above the standard error under some experimental conditions. The proteolytic sensitivity of the A domain of Toc159 led to the analysis of complexes containing the 86 kDa fragment. The degradation of this protein is far from a novel observation, as this protein was known as Toc86 for many years. Protein translocation into chloroplasts has been characterized with in vitro import assays into *Pisum sativum* chloroplasts containing Toc159 mostly as the 86 kDa fragment (Jarvis 2008). Degradation leads to a slight decrease in import efficiency, but many insights into the mechanism of translocation have been reached through this type of experiment. Kikuchi et al. (2006) showed a decrease in complex size correlated to loss of the A domain using BN-PAGE. Two studies have demonstrated that in order to purify chloroplasts containing non-proteolyzed Toc159, the protocol for chloroplast isolation must be optimized to 15-20 minutes, rather than 40 minutes or more, and the isolation medium must be maintained at 0ºC to 2ºC for the entirety of the protocol in the presence of a protease inhibitor cocktail (no. P-9599, Sigma) (Bolter, May et al. 1998; Chen, Chen et al. 2000). These conditions were followed as closely as possible, but incubation at higher temperature was necessary for efficient incubation/NT exchange, so proteolysis was inevitable, even with the inclusion of protease inhibitors and optimization of chloroplast isolation protocol for speed. Experiments utilizing chloroplasts of differing ages showed increased proteolysis

of Toc159 in older plants, most likely due to the increased presence of cytosolic or plastidic proteases during development. One other problem that may have been encountered was the presence of residual nucleotide triphosphates, even after dark conditioning and maintaining chloroplasts on ice (with the exception of incubations carried out at 25ºC). The possible presence of residual nucleotide triphosphates increases the difficulty of data interpretation. Several studies have suggested that homo or heterodimeric interactions between the Toc GTPases are modulated by their energetic state. Different experimental setups have yielded seemingly contradictory results, which emphasizes the complex nature of the interactions between the Toc components and their association partners. Nucleotide induced instability or oligomerization of the Toc complex is an interesting area of research, and more research is necessary to identify the mechanisms that underlie oligomerization of the Toc complex.

3.4.3 Future Directions

In order to further characterize the stability/oligomerization of the Toc complex by BN-PAGE and second dimension SDS-PAGE, several steps could be taken to increase the effectiveness of this assay. Chloroplasts always contain residual NTPs and NDPs, and due to the action of myokinase and nucleoside diphosphate kinase, during the course of an experiment every type of NTP can be produced or interconverted (Soll and Schleiff 2004). In order to eliminate or at least minimize endogenous NTPs and NDPs chloroplasts could be incubated with apyrase and nigericin before nucleotide incubations or binding experiments. Apyrase is a membrane-impermeant ATP degrading enzyme with ATPase, ADPase, and GTPase activity which can be used to deplete NTPs in the medium outside of chloroplasts (Olsen and Keegstra 1992). The ionophore Nigericin (H^+/K^+ antiporter) can be used to uncouple electron transport from ATP synthesis, depleting endogenous ATP (Olsen, Theg et al. 1989; Theg, Bauerle et

al. 1989). The presence and ratio of residual nucleotides has a large effect on the effectiveness of AlF_4 to function as a transition state analog. Therefore, future experiments using this transition state analog should take care to deplete external NTPs with apyrase and add exogenous GDP and Mg^{2+} with AlF_4.

In the present study, the detergent to chloroplast ratio was normalized to chlorophyll concentration. Chlorophyll is accumulating during development, so if plants of slightly different ages are used in separate experiments, the detergent to chlorophyll ratio can be the same, but the detergent to Toc ratio may actually be different. Normalization of detergent concentration to envelope surface area would be ideal in order to minimize variation between experiments, but this would be a very difficult measurement to make. One possible solution would be to normalize detergent concentration to the number of chloroplasts, determined with a hemocytometer. Pea plants used to prepare chloroplasts were not exposed to light on the day that they were used, but chloroplast preparation did not always occur at the same time of day. Therefore, plants underwent different periods of dark treatment for different experiments. In the future, preparation of chloroplasts at a set time of day may reduce the amount of variation between experiments.

There has been some controversy over the orientation of Toc75, and several models have been proposed (Hinnah, Hill et al. 1997; Sveshnikova, Grimm et al. 2000). Both models agree on the presence of 16 membrane-spanning β-strands with an N_{in}-C_{in} orientation; however, the models disagree on the location of several loop regions, including the putative POTRA domains. The POTRA domains could be cytosolically exposed or could be in the intermembrane space. If they are located in the intermembrane space, the experimental strategy utilized here would probably not differentiate between a chaperone-like or structural role. In order to investigate this possibility, chloroplast envelopes could be purified as inside-out vesicles, incubated with isolated POTRA domains, solubilized and analyzed by 2d PAGE. Utilizing isolated envelopes would result in increased proteolytic degradation of Toc159

due to the time consuming protocol, resulting in some dissociation of Toc components; however, this procedure may be able to differentiate between the proposed roles of the POTRA domains.

A change in the model organism from which chloroplasts are isolated would expand the possible experiments that could be investigated with the general experimental strategy used in the present study. Utilizing chloroplasts purified from the model organism *Arabidopsis thaliana* would expand the genetic manipulations that could give insight into Toc complex dynamics, and the repercussions of the presence of multiple isoforms of the GTPase receptors on Toc complex assembly could be examined. The structural implications of the *ppi1* and *ppi2* mutant lines on Toc complex oligomeric status could also be investigated. Generation of antibodies able to differentiate between the isoforms of the GTPases could give insight into the influence of the various components on Toc complex assembly.

CHAPTER IV
4.0 TIRF MICROSCOPY OF TOC COMPLEX

4.1 Introduction

Total internal reflection fluorescence (TIRF) microscopy has been used for more than 25 years to study cellular processes occurring near the plasma membrane. When the angle of the excitation beam is greater than the critical angle of refraction, the beam is totally reflected by the interface, producing an evanescent wave normal to the surface that decays exponentially with distance perpendicular to the surface. This allows for the excitation of fluorophores in close proximity to the surface, while minimizing the excitation of distal fluorophores and autofluorescence (Axelrod 2001). For typical experiments, identical fluorophores located 1, 10, 100, and 1000 nm from the surface will emit relative fluorescent intensities of 0.99, 0.92, 0.43, and 0.0002, respectively (Axelrod 1981). Therefore, TIRF microscopy is used to selectively excite fluorophores very near a solid surface (≤100 nm, compared to ~500-800 nm optical section for confocal systems), generating images with very low background fluorescence and a high signal to noise ratio, unmatched by any other light microscopy technique (Toomre and Manstein 2001). TIRF microscopy allows for the monitoring of individual biomolecules in their native environment in real time, as well as the study of their dynamics and kinetics, via labeling with fluorophores such as sulphoindocyanine dyes (Cy3 and Cy5), sulphonated rhodamine dyes (Alexa532 and Alexa546), or the genetic incorporation of enhanced GFP (Toomre and Manstein 2001).

4.2 Results

In order to investigate the mobility of individual Toc complexes, isolated intact chloroplasts were immunodecorated with Alexa488 conjugated antibodies against Toc159 and visualized with a Nikon Laser TIRF system in brightfield and subsequently deconvoluted, Fig. 4-1 A and B, respectively. TIRF microscopy allows for the visualization of individual Toc complexes on the chloroplast surface, while minimizing the background chlorophyll autofluorescence. The antibodies most likely bind to one Toc159 molecule, as bridging between two Toc core complexes is theoretically possible, but unlikely and sterically unfavorable, as the binding epitope is most likely buried and the two complexes would be required to be very close in a particular orientation. NIS Elements software was utilized for particle tracking and analysis. Panels C, D, and E in Fig. 4-1 represent the velocity and acceleration with respect to time for selected single fluorescent particles on separate chloroplasts. The motion of the single particles is discontinuous, characterized by long periods of stasis followed by bursts of movement.

4.3 Discussion and Future Directions

The results presented here provide an encouraging proof of principle, showing that TIRF microscopy can be utilized to investigate the Toc complex in its native membrane. In the future, the use of multicolor immunolabeling will allow the simultaneous tracking of all of the Toc components, allowing the monitoring of the association/dissociation dynamics of the individual Toc components. The inclusion of preproteins and/or nucleotide could be used to investigate the effects of energetics and translocation status on Toc complex

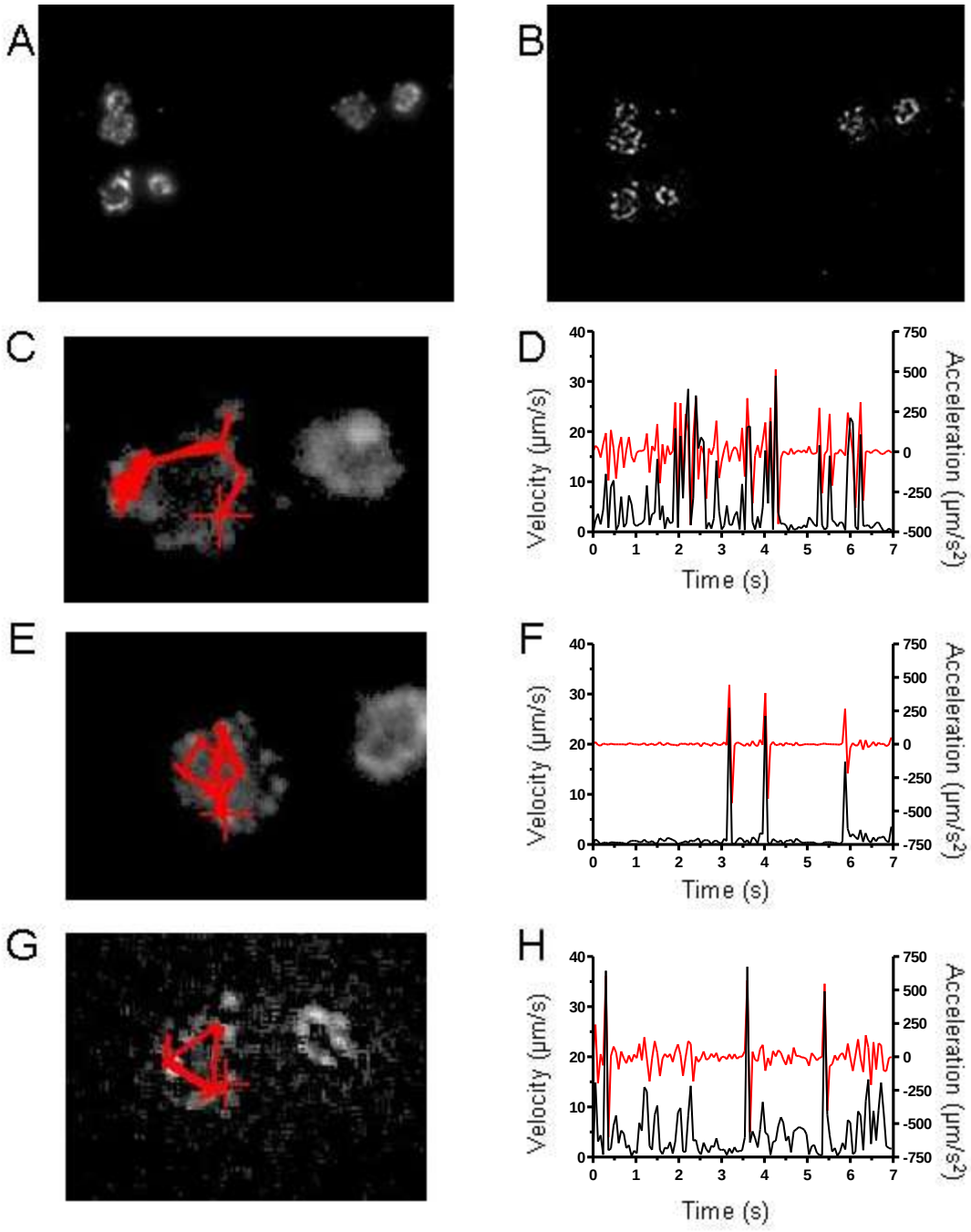


Figure 4-1 Tirf microscopic analysis of Toc159 dynamics.
A and B represent wide field image of isolated chloroplasts immunodecorated with α-Toc159 antibodies. B Dynamic background subtraction with Nikon NIS software results in deconvoluted image. C, D, & E represent chloroplasts on which one fluorescent spot has been selected and tracked, yielding the acceleration and velocity measurements with respect to time in the graphs to the right.

mobility and composition. If chloroplasts were used from *Arabidopsis thaliana*, a plethora of genetic studies could utilize TIRF microscopy. The generation of antibodies to the multiple isoforms of the GTPase receptors would allow the investigation of the composition of multiple, substrate specific translocons, and their possible differences in mobility or dynamics. Mutant chloroplasts from the *ppi1* and *ppi2* lines (null mutants for atToc33 and atToc159, respectively) could be utilized to investigate the effects of the absence of a major Toc subunit on complex assembly. In *Arabidopsis*, studies utilizing GFP could be undertaken, such as the attachment of GFP to Toc159, possibly in place of the A domain. The utilization of GFP rather than fluorophore conjugated antibodies would result in a much smaller mass associated with the receptor, which could yield mobility more relevant to the *in vivo* state, although this GFP integration site would most likely have structural consequences. If the site of proteolytic cleavage was identified and altered, GFP could be attached at the N-terminus of the A domain, allowing for visualization of full size Toc complexes by TIRF microscopy.

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APPENDIX

Supplemental Figures

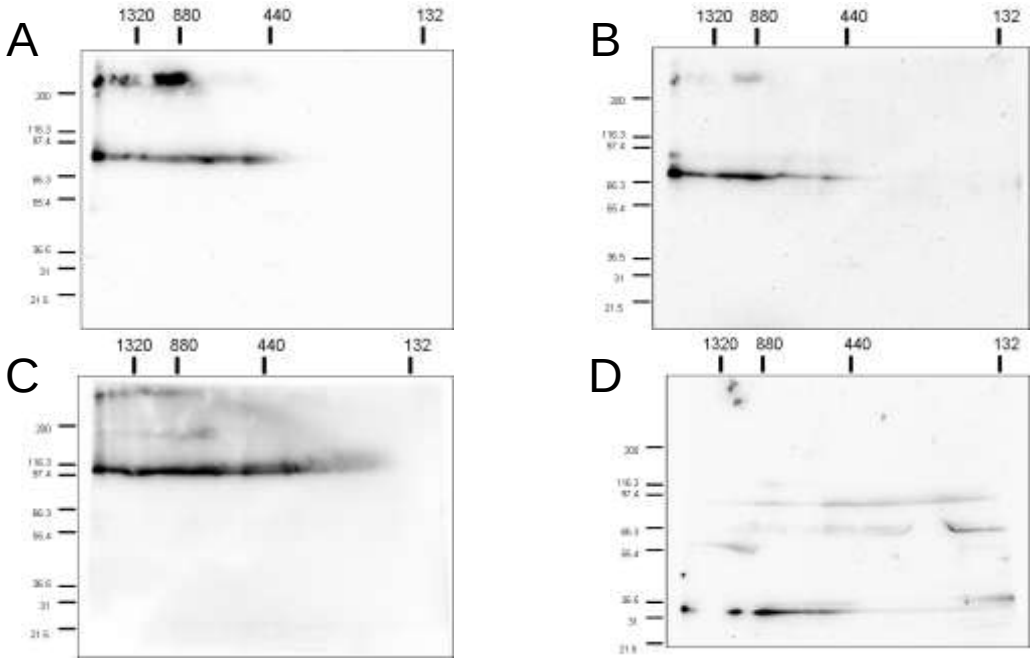

Figure S-1 Two-dimensional BN/SDS-PAGE analysis of Tic110 distribution. Blots are control treatment of chloroplasts from first POTRA experiment. A represents the α-Toc159 western blot. B represents the PVDF from A stripped and reprobed with α-Toc75 antiserum. C represents the PVDF from A & B stripped and reprobed with α-Tic110 antiserum. D represents a separate 2d gel and western blot with α-Toc34 antiserum.

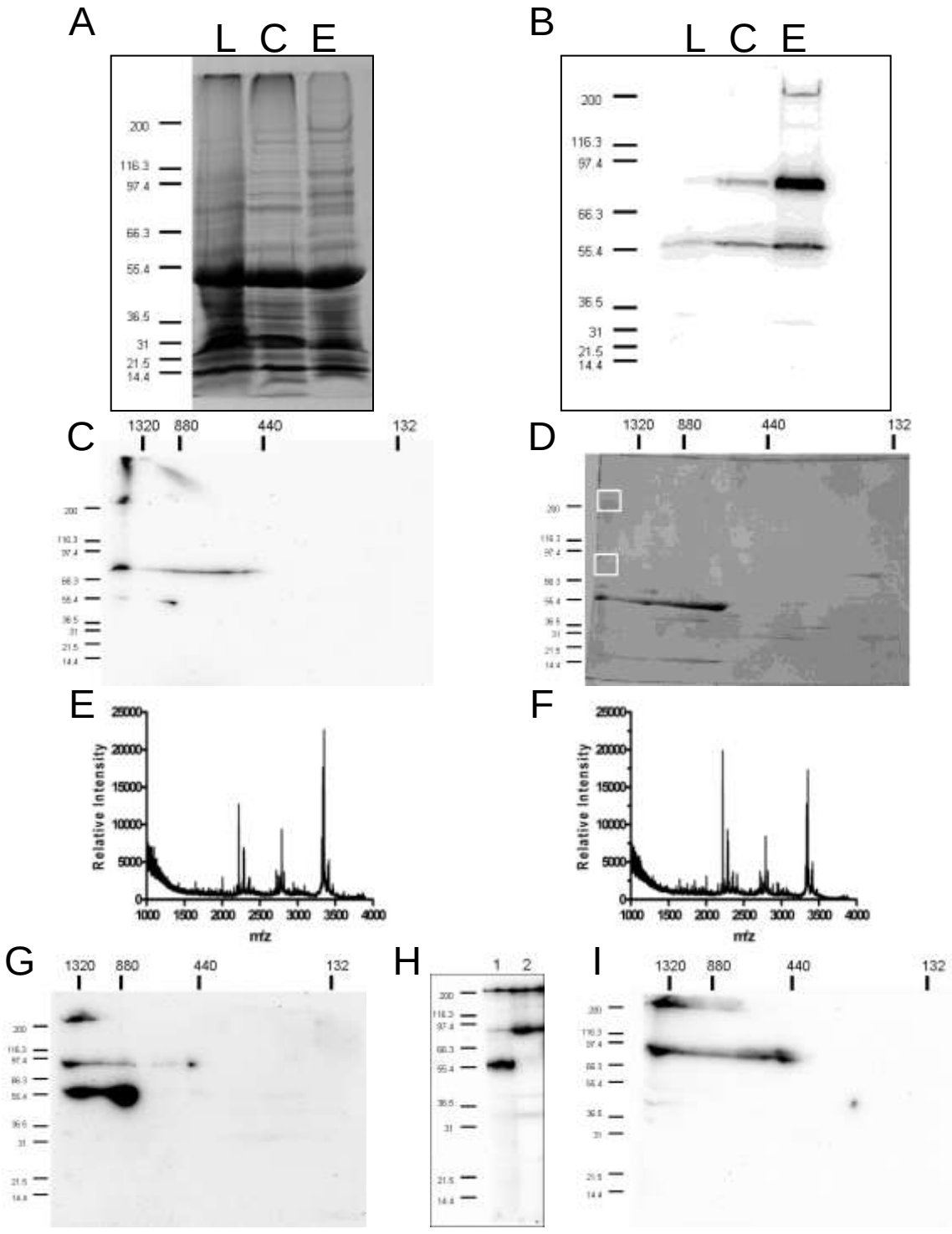
A
L C E
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
B
L C E
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
C
1320 880 440 132
D
1320 880 440 132
E
Relative Intensity
m/z
F
Relative Intensity
m/z
G
1320 880 440 132
H
1 2
I
1320 880 440 132
J
A-domain
(1-598)
G-domain
(599-1062)
M-domain
(1063-1499)

Figure S-2 Investigation of Toc159 degradation.
Panels A and B represent the Coomassie stain and western blot (α-Toc86 from Danny Schnell), respectively, of whole pea leaf extract, and pea chloroplasts and envelopes isolated in the presence of a protease inhibitor cocktail (PIC, Sigma P-9599). Purified envelopes were analyzed by BN-PAGE and 2d SDS-PAGE, with the α-Toc86 western blot in C and Coomassie stained gel in D. Spots represented by the white squares in D were excised, in gel digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. E and F represent the MALDI spectra for the 86 kDa species and the 159 kDa species, respectively. G represents the western blot of the 2d electrophoretic analysis of Toc159 with α-Toc86 antiserum from Danny Schnell. H represents the western blot analysis of chloropasts purified in the presence of PIC, with α-Toc86 from Danny Schnell and antiserum against the M domain from Masato Nakai in lanes 1 and 2 respectively. G represents the western blot of the 2d electrophoretic analysis of Toc159 with α-Toc159(M) antiserum from Masato Nakai. J represents the proposed tripartite structure of psToc159 (adapted from Chen *et al.* 2000).

Table S-1 Comparison of observed fragments to predicted for MALDI of the 86 kDa species.

A mass list was generated from the spectra in Fig. S-2 E and was compared to a list of fragments produced by an *in silico* trypsin digestion using the PROWL suite of applications available at http://prowl.rockefeller.edu/

86 kDa species

Fragment	Residues		Mass	Measured Mass
120-124	1300-1316	K/Q...R/N	2098.480	2096.612+/-3.00
99-103	1140-1156	R/K...K/Q	2187.568	2186.580+/-3.00
92-93	1068-1086	R/A...K/L	2187.617	2186.580+/-3.00
98-101	1134-1151	K/L...K/L	2274.519	2274.554+/-3.00
98-101	1134-1151	K/L...K/L	2274.519	2275.514+/-3.00
98-101	1134-1151	K/L...K/L	2274.519	2276.471+/-3.00
104-111	1157-1173	K/Q...K/N	2275.735	2274.554+/-3.00
104-111	1157-1173	K/Q...K/N	2275.735	2275.514+/-3.00
104-111	1157-1173	K/Q...K/N	2275.735	2276.471+/-3.00
10	126-147	K/G...R/E	2279.360	2276.471+/-3.00
103-111	1156-1173	K/K...K/N	2403.909	2401.410+/-3.00
26	396-423	R/V...K/D	2874.964	2874.615+/-3.00
100-105	1141-1163	K/A...K/R	3029.490	3029.449+/-3.00
75-77	910-935	K/S...R/S	3086.585	3083.676+/-3.00
137-139	1426-1456	K/L...K/N	3311.915	3310.866+/-3.00
137-139	1426-1456	K/L...K/N	3311.915	3311.794+/-3.00
137-139	1426-1456	K/L...K/N	3311.915	3312.785+/-3.00
99-106	1140-1164	R/K...R/M	3313.852	3310.866+/-3.00
99-106	1140-1164	R/K...R/M	3313.852	3311.794+/-3.00
99-106	1140-1164	R/K...R/M	3313.852	3312.785+/-3.00
121-126	1307-1337	R/G...K/L	3315.779	3312.785+/-3.00
44-45	673-707	K/E...R/L	3335.631	3337.997+/-3.00
31-32	487-518	K/S...K/V	3340.517	3337.997+/-3.00
31-32	487-518	K/S...K/V	3340.517	3338.886+/-3.00
31-32	487-518	K/S...K/V	3340.517	3339.859+/-3.00
31-32	487-518	K/S...K/V	3340.517	3340.757+/-3.00
68-74	880-909	R/V...K/S	3400.968	3402.381+/-3.00
99-107	1140-1166	R/K...R/D	3601.238	3601.746+/-3.00
98-105	1134-1163	K/L...K/R	3855.414	3853.172+/-3.00
86-90	1031-1065	K/P...R/S	4013.743	4015.814+/-3.00
138-140	1434-1469	R/T...Y/-	4015.620	4015.814+/-3.00
45-49	686-732	K/A...R/P	4567.058	4569.114+/-3.00
116-119	1255-1299	K/F...K/Q	4761.308	4761.417+/-3.00
113-115	1214-1254	R/Y...K/F	4763.307	4761.417+/-3.00
47-56	714-769	R/P...K/Y	6062.004	6059.420+/-3.00
127-134	1338-1415	K/L...K/M	8438.425	8436.026+/-3.00

Table S-2 Comparison of observed fragments to predicted for MALDI of the 159 kDa species.

A mass list was generated from the spectra in Fig. S-2 F and was compared to a list of fragments produced by an *in silico* trypsin digestion using the PROWL suite of applications available at http://prowl.rockefeller.edu/

159 kDa species

Fragment	Residues		Mass	Measured Mass
105-111	1160-1173	R/E...K/N	1805.203	1804.400+/-3.00
104-108	1157-1169	K/Q...K/K	1806.147	1804.400+/-3.00
95-99	1125-1140	K/P...K/A	1838.185	1840.403+/-3.00
61-62	800-816	R/Q...R/L	1840.045	1840.403+/-3.00
99-103	1140-1156	R/K...K/Q	2187.568	2186.909+/-3.00
92-93	1068-1086	R/A...K/L	2187.617	2186.909+/-3.00
98-101	1134-1151	K/L...K/L	2274.519	2273.481+/-3.00
98-101	1134-1151	K/L...K/L	2274.519	2274.419+/-3.00
98-101	1134-1151	K/L...K/L	2274.519	2275.442+/-3.00
98-101	1134-1151	K/L...K/L	2274.519	2276.367+/-3.00
98-101	1134-1151	K/L...K/L	2274.519	2277.337+/-3.00
104-111	1157-1173	K/Q...K/N	2275.735	2273.481+/-3.00
104-111	1157-1173	K/Q...K/N	2275.735	2274.419+/-3.00
104-111	1157-1173	K/Q...K/N	2275.735	2275.442+/-3.00
104-111	1157-1173	K/Q...K/N	2275.735	2276.367+/-3.00
104-111	1157-1173	K/Q...K/N	2275.735	2277.337+/-3.00
10	126-147	K/G...R/E	2279.360	2276.367+/-3.00
10	126-147	K/G...R/E	2279.360	2277.337+/-3.00
26	396-423	R/V...K/D	2874.964	2874.700+/-3.00
137-139	1426-1456	K/L...K/N	3311.915	3311.735+/-3.00
99-106	1140-1164	R/K...R/M	3313.852	3311.735+/-3.00
31-32	487-518	K/S...K/V	3340.517	3338.866+/-3.00
31-32	487-518	K/S...K/V	3340.517	3339.697+/-3.00
31-32	487-518	K/S...K/V	3340.517	3340.480+/-3.00
68-74	880-909	R/V...K/S	3400.968	3402.519+/-3.00
98-105	1134-1163	K/L...K/R	3855.414	3853.836+/-3.00
138-140	1434-1469	R/T...Y/-	4015.620	4018.361+/-3.00
45-49	686-732	K/A...R/P	4567.058	4568.703+/-3.00
116-119	1255-1299	K/F...K/Q	4761.308	4761.188+/-3.00
113-115	1214-1254	R/Y...K/F	4763.307	4761.188+/-3.00
127-134	1338-1415	K/L...K/M	8438.425	8438.788+/-3.00

VITA

William Ikard Crenshaw was born in Winchester, Tennessee on November 12, 1982. His elementary education was conducted at Townsend, Mary Sharpe, and Clark Memmorial elementary schools. He attended South Junior High and graduated from Franklin County High School in 2001. He obtained his Bachelor's Degree in Biology from Birmingham-Southern College in Birmingham, Alabama in 2005. He joined the graduate program in Biochemistry and Cellular and Molecular Biology in the fall of 2005. He joined the lab of Barry D. Bruce in the spring of 2006 to conduct research on the translocation apparatus in the outer chloroplast membrane of *Pisum sativum*. In the spring of 2009, he completed the Professional Brewers Certificate Program at the University of California, Davis, and was elected as a Member of the Institute of Brewing & Distilling and the Master Brewers Association of the Americas.